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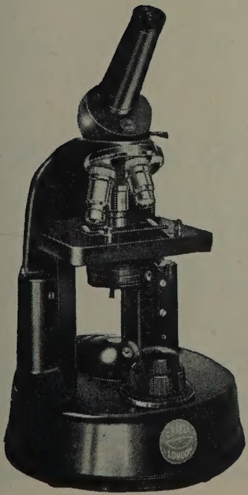

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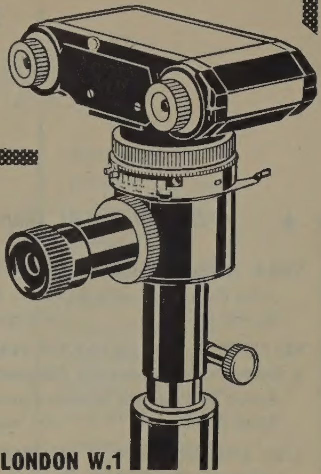

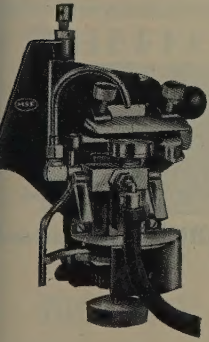

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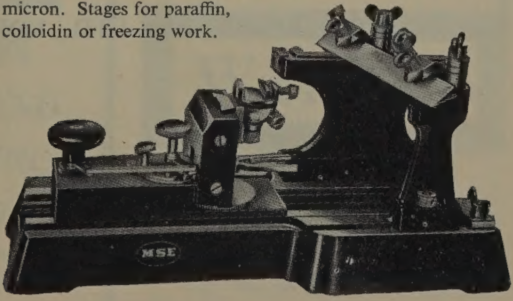

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Axon Structure and the Dictyosomes (Golgi Bodies) in the Neurones of the Cockroach, *Periplaneta americana*

By V. B. WIGGLESWORTH

(*From the A.R.C. Unit of Insect Physiology, Department of Zoology, University of Cambridge*)

With two plates (figs. 1 and 2)

SUMMARY

The large axons in *Periplaneta* are composed of bundles of osmiophobe strands about 0·5 μ thick which fan out into the body of the nerve-cell. These strands are here termed 'neurofibrils'; it is suggested that the dictyosomes (Golgi bodies) are concerned in their secretion.

The dictyosomes are well stained by the osmium and ethyl gallate method. Each dictyosome surrounds or is applied to an unstained canal which runs into the cytoplasm and is believed to be continuous with the 'neurofibrils' as defined. There are all intermediate stages between thin fusiform sheaths of osmiophil material around the 'neurofibrils' and the thick rings or cuffs which form the dictyosomes. The clear canals through the dictyosomes are arranged in the main concentrically around the nucleus in the body of the cell; they commonly converge upon the nerve-fibre in the axon cone.

THE purpose of this study is to consider possible connexions between the dictyosomes or Golgi bodies and the structural organization of insect axons. The neurones are exceedingly rich in lipids which undoubtedly play a major role in maintaining the cell structure. Earlier work has shown that the stabilization of lipid membranes by fixation with buffered osmium tetroxide, followed by visualization of the bound osmium with ethyl gallate (Wigglesworth, 1957, 1959 *a*, *b*), reveals structural details that are not readily seen by standard histological methods.

The last abdominal ganglion of the adult male cockroach, *Periplaneta americana* (L.), has been used for most of the observations, the third thoracic ganglion for some. Sections were cut at 0·5 to 2 μ (Wigglesworth, 1959*c*). Some observations on the glial invaginations (Holmgren's canals or 'trophospongium') of the large ganglion cells are being published elsewhere (Wigglesworth, 1960). The fine structure of cockroach ganglia as seen with the electron microscope is described by Hess (1958).

Structure of the large axons

The large axons of insects as seen in silver preparations appear to be made up of a number of neurofibrils which spread out in a fan-like manner in the cell-body to form a meshwork around the nucleus (Beams and King, 1932). But in silver preparations the cytological structure is so greatly impaired that the real existence of these neurofibrils has been a frequent subject of controversy.

The axons in *Rhodnius* as seen in sections stained with osmium and ethyl

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gallate (Wigglesworth, 1959*b*) have a rope-like structure and consist of unstained neurofibrils which fan out to form an investment of the nucleus on entering the cell-body. The existence of such neurofibrils is even more evident in the cockroach.

Giant axons arise from large cells in the last abdominal ganglion and run to the opposite side, forming a conspicuous chiasma where they intersect and cross over (fig. 1, A). These axons are about 22 μ thick. As seen in longitudinal section they consist of a great number of uniform unstained fibrils about 0·5 μ in thickness, which run a more or less wavy course along the axon. The matrix which fills the spaces between the fibrils is only slightly more osmiophil than the fibrils themselves, but this interfibrillar material stains sufficiently darkly to show up the white strands between. The whole structure resembles a bundle of cooked spaghetti with raisins in the form of mitochondria lying at intervals between the strands. Where the axon divides, a greater or smaller number of fibrils separate from the main bundle to form the branch. Where the axon comes off the cell-body the neurofibrils sometimes follow an oblique course which gives the whole fibre a rope-like appearance (fig. 1, E).

In transverse sections it is possible to see the fibrils very faintly as clearer holes in a very pale grey matrix. It is evident that the fibrils are not bounded by a continuous lipid membrane, which would define their limits much more clearly. It is doubtless for this reason that they do not show up well in electron micrographs.

There can be no doubt that these neurofibrils are constant structural components of the axons. They can be readily seen in smaller axons (fig. 1, B) down to a diameter of 3 μ or less; the individual fibrils remain about the same size throughout.

The axon is the product of the cell-body, and there is now good evidence that every axon is being continuously renewed by secretion from its ganglion cell (Koenig, 1958; Weiss, 1959). It would seem probable that there must be some organized structure within the cell to produce this degree of organization in the nerve-fibre.

The course of neurofibrils in the ganglion cell

When attempting to follow the neurofibrils into the body of the ganglion

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FIG. 1 (plate). All 2-μ sections, osmium tetroxide, and ethyl gallate.

A, giant axons crossing in mid-line, showing neurofibrils and mitochondria.

B, oblique section of axon showing neurofibrils.

C, transverse section of ganglion cells and base of axons, showing multiple invaginations by the glial cells.

D, oblique longitudinal section of base of axon, showing glial invaginations giving a false impression of neurofibrils.

E, ganglion cell showing rope-like neurofibrils in the axon traceable into the cell-body; dictyosomes converging on the axon.

F, dark type of ganglion cells with Golgi bodies; the cell at top left shows neurofibrils from axon dispersing in the cell.

G, dark and pale types of ganglion cells; the dark cells show clear canals (? neurofibrils) in the cytoplasm.

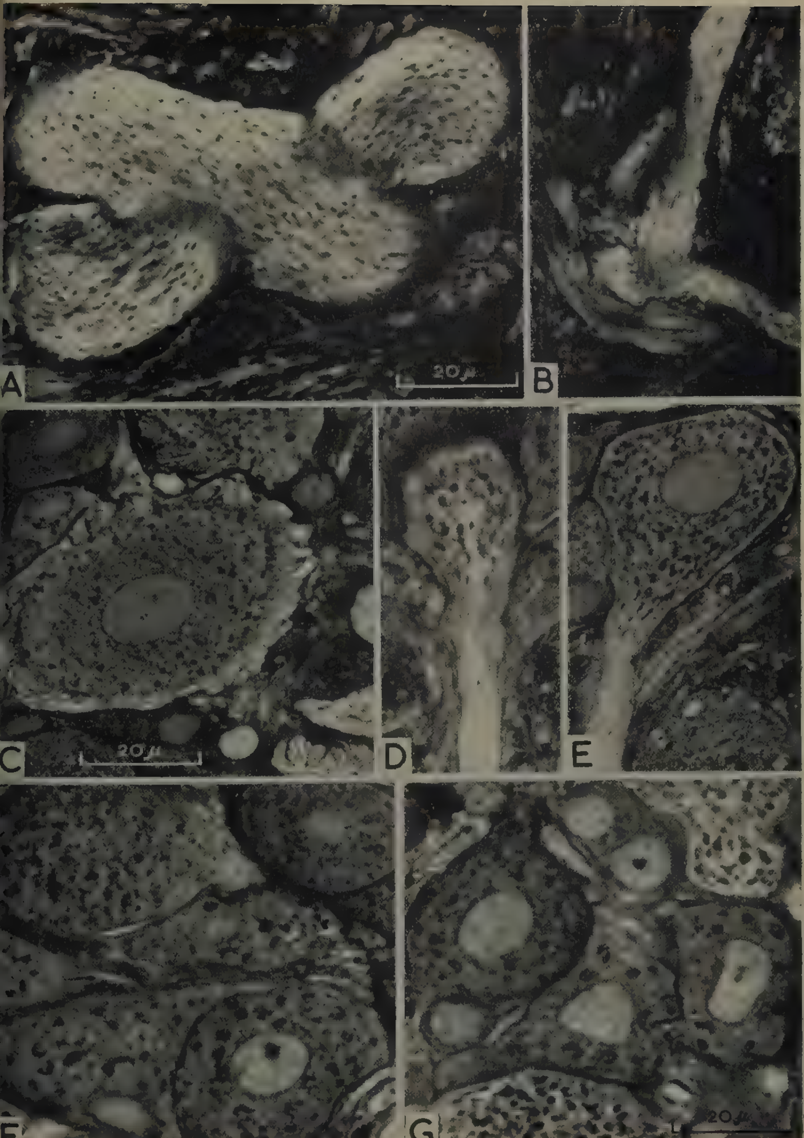

FIG. 1

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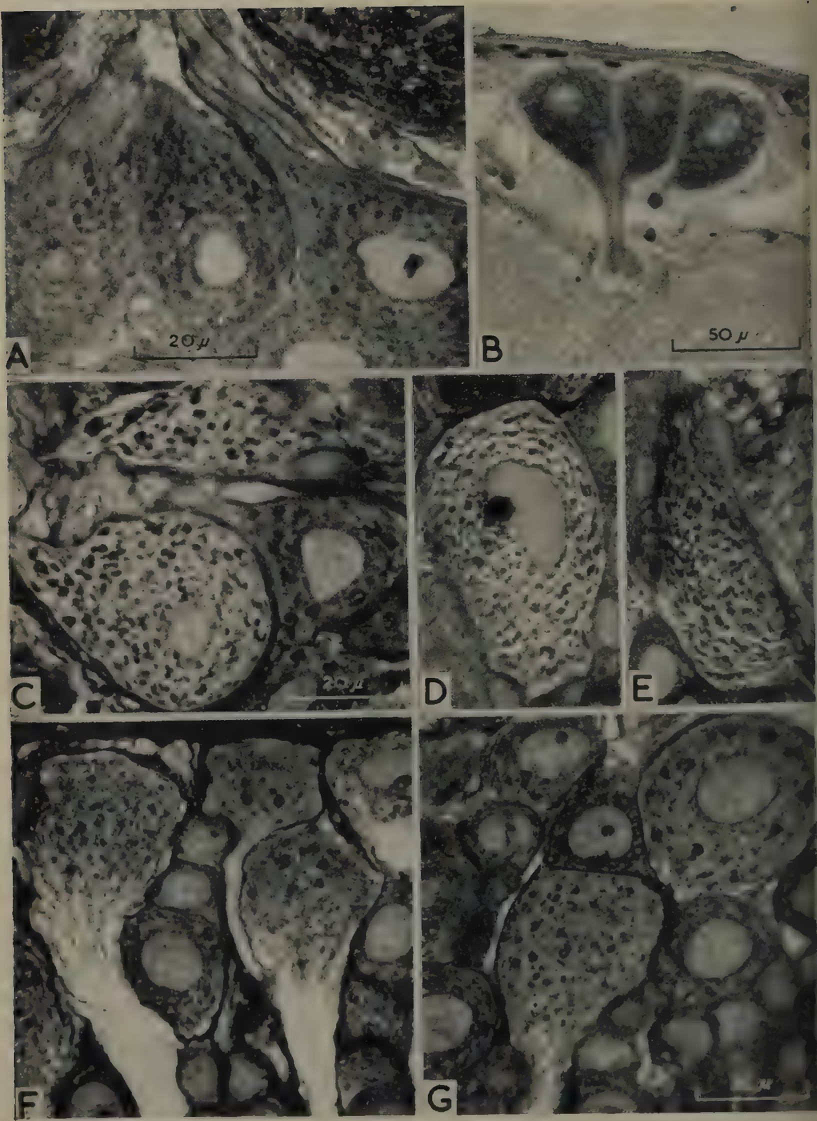

FIG. 2

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cell it is easy to be misled by the glial invaginations. As described elsewhere (Wigglesworth, 1960) these invaginations become progressively developed towards the base of the axon. Transverse sections at this level show these invaginations as radially disposed flanges or laminae extending far into the cell (fig. 1, C). Longitudinal sections in this region cut in a slightly eccentric plane can produce an appearance which superficially resembles a bundle of neurofibrils (fig. 1, D). But, as already pointed out, the neurofibrils are not bounded by lipid sheaths such as form the walls of the glial invaginations. They are far less conspicuous structures than these 'pseudofibrils'.

None the less it is often possible to follow the true neurofibrils into the cell. They sometimes fan out evenly in all directions. Sometimes they follow a zigzag course. It is seldom possible to trace individual fibrils very far; they are soon lost among the granular contents of the cytoplasm (figs. 1, E, F; 2, A, G).

As pointed out by Hess (1958) the ganglion cells of the cockroach as seen with the electron microscope are of two sorts, dark cells and pale cells, depending on the density of the cytoplasm. These two kinds of ganglion cell are equally distinct after staining with osmium and ethyl gallate (figs. 1, G; 2, C). It is often difficult to detect the colourless neurofibrils in the cytoplasm of the pale cells; but in the dark cells they can readily be seen. The cell may sometimes appear filled with these fine, convoluted, unstained, worm-like structures (figs. 1, G; 2, C). If the cell is cut at right angles to the line of entry of the axon it is possible, by changing the plane of focus on a 2-μ section, to follow the fibrils up and down. They are not unlike the pore canals as seen in a tangential section of the cuticle, but follow a much less regular course.

The dictyosomes (Golgi bodies) and the neurofibrils

The object in following the neurofibrils in the body of the ganglion cell was to seek some visible structure which might be concerned in their secretion. It seems probable that the dictyosomes or Golgi bodies are involved.

The Golgi bodies are very evident in the cytoplasm of the ganglion cells as darkly staining objects varying from 0·75 μ to 3·0 μ across (fig. 1, F, G). They take the form of crescents, horse-shoes, parallel bars, or rings. For the most part they stain a deep brownish grey or black with osmium and ethyl gallate, which suggests that phospholipids are largely responsible for binding the osmium. But not uncommonly they have a thin outer shell which stains

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FIG. 2 (plate). A, ganglion cells with Golgi bodies oriented towards base of axon; neurofibrils well seen in the cell to the left.

B, 4-μ section of ganglion cells; alcoholic Bouin and toluidine blue. Dictyosomes appear as clear spaces with RNA deposits between.

C, pale and dark ganglion cells; the dark cell to the right shows worm-like canals (?neurofibrils) in the cytoplasm; the pale cell to the left shows dictyosomes oriented towards the axon.

D, pale cell showing concentric arrangement of dictyosomes.

E, similar cell to D with Golgi bodies oriented towards the axon (above).

F, ganglion cells 5 days after section of nerve, showing disorganization of axons.

G, corresponding cells on the normal side of the same insect.

a uniform or granular blue black and is perhaps composed of triglycerides (fig. 3, A).

Every Golgi body is associated with a colourless unstained object or 'vacuole'. The osmiophil substance may be applied to this in the form of a crescent, but usually surrounds it as a more or less complete ring. By focusing up and down it is generally possible to see that the colourless part of the Golgi body is a clear canal and that this becomes continuous with a similar canal which is soon lost in the cytoplasm. The dimensions of these canals are about the same as those of the neurofibrils.

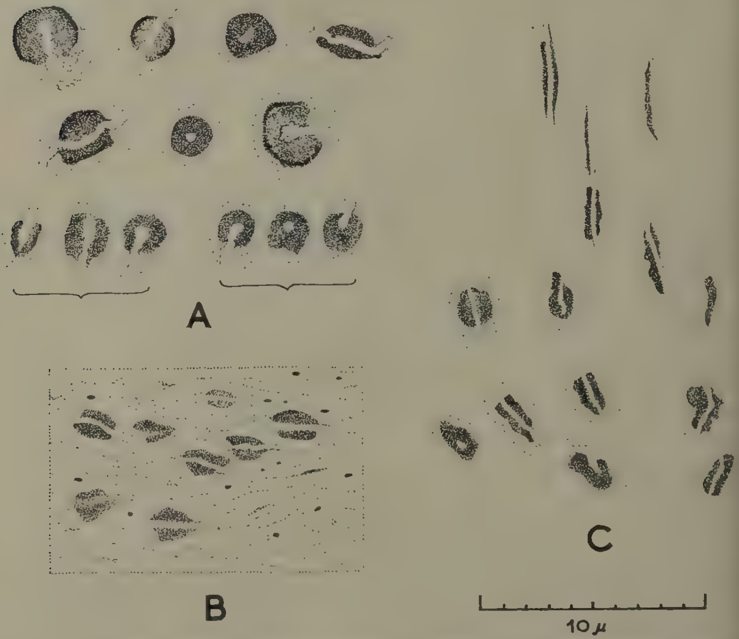

FIG. 3. A, series of typical dictyosomes from the group of dark ganglion cells shown in fig. 1, F, G. The bracketed groups below represent single dictyosomes as seen at three levels of focus.

B, detail of dictyosomes and mitochondria seen in the axon cone of the cell to the left of fig. 2, A. The axon lies to the right of the drawing.

C, selected dictyosomes from the pale cell shown in fig. 2, E. The axon lies beyond the upper part of the drawing. The dictyosomes have their actual orientation; those above were in the axon cone, those below in the body of the cell.

It is difficult to avoid the impression that the Golgi bodies are in fact deeply staining cuffs around unstained canals which become continuous with the neurofibrils of the axons. The evidence in support of this interpretation may be formulated as follows:

(i) The Golgi bodies may sometimes include slightly larger vacuoles, but in nearly all of them the canals which they enclose are of the same size as the neurofibrils.

(ii) In large ganglion cells in which the Golgi bodies are very numerous, notably in the large pale cells which give rise to giant axons, the Golgi bodies have a characteristic orientation which agrees with the orientation of the neurofibrils. Throughout the greater part of the body of the cell they lie with the lumen, that is with the long axis, disposed concentrically around the nucleus (fig. 2, D); whereas in the axon cone they converge upon the base of the axon (figs. 1, E; 2, A, E; 3, B, C).

(iii) Where the neurofibrils are entering the base of the axon they are often enclosed in thin elongated cuffs of osmiophil material which taper away at both ends. Cuff-like structures of this kind may be seen radiating out from the base of the axon, and all intermediate stages between them and typical dictyosomes can be found (fig. 3, C). This suggests that the typical dictyosome results from the increasing concentration of this diffuse osmiophil covering into discrete points on the fibril. Such an interpretation would account for the great diversity in the form of the bodies in different ganglion cells.

(iv) An attempt was made to observe the changes in the Golgi bodies during axon regeneration. The crural nerve (nerve 5 of Pringle, 1939) runs immediately below the very thin cuticle at the base of the coxa. It is easy to make an incision at this point and to cut the nerve without injury to the tracheal system. This operation was performed on the left metathoracic leg in a dozen adult cockroaches about one month old.

The crural nerve is mainly a sensory nerve, but it does contain some 50 motor axons of 3 to 10 μ diameter. After section the sensitivity of the tarsus is lost and that leg is not used in walking. But, as Bodenstein (1957) has shown, regeneration takes place and motor activity is being recovered by 6 weeks after the operation.

As in *Rhodnius* (Wigglesworth, 1959*b*) the motor axons form two groups, with thin sheaths and with thick sheaths. Some at least of the motor axons of nerve 5 after entering the metathoracic ganglion run inwards and ventrally to end in a group of ganglion cells on the same side, which lie just a little anterior to the point of entry of the nerve, and are very readily compared with the corresponding cells of the opposite side.

These two groups of cells were compared at different periods after section of the nerve. At 5 days after section the ganglion cells on the operated side had their axons distended and apparently disorganized with no distinct neurofibrils visible (fig. 2, F). Within the cytoplasm of the cell-body the canals associated with the Golgi bodies were more dilated than on the control side.

At 3 weeks and 6 weeks after nerve section, when the movements of the muscles showed that regeneration was in progress, the cells on the operated side showed several differences from those on the control side. The nucleoli were greatly enlarged. The cytoplasm was more deeply stained, and close examination showed that this staining largely took the form of a diffuse dark sheath around the clear canals that are presumed to be neurofibrils. This darkening was associated with a darker staining of the Golgi bodies.

It looks as though during the regeneration of the axons there is an increase in the activity of the Golgi bodies.

Nucleic acid in the ganglion cell

It has been shown recently by Malhotra (1959) that cytoplasmic nucleic acid in the neurones of vertebrates has the same distribution as the Golgi apparatus and he concludes that in this material the Golgi structure results from the deposition of silver or osmium on the Nissl bodies. In the neurones of *Helix* Boyle (1937) found that the Nissl substance occurs in fine flocculent granules dispersed throughout the cytoplasm. A similar distribution of ribonucleic acid was observed by Shafiq and Casselman (1954) in the neurones of *Locusta*.

These results have been confirmed in *Periplaneta*. Ganglia were fixed with alcoholic Bouin solution and sections stained with toluidine blue, pyronin / methyl green, and gallocyanin before and after incubation with ribonuclease. RNA was present in the form of fine granules dispersed throughout the cytoplasm between the dictyosomes with a slight increase in density around the nucleus and possibly around the surface of the dictyosomes. The dictyosomes themselves appear as clear rounded spaces devoid of RNA (fig. 2, B).

Discussion

According to current ideas the Golgi apparatus is concerned in some way with the later stages of the process of secretion in the cell. That has indeed been the opinion of classical cytologists for many years. The most suggestive evidence has been provided by a study, with the light microscope and with the electron microscope, of the cycle of secretion in the mammalian pancreas (Hirsch, 1958, 1959).

Whether the dictyosomes in the nerve-cells of invertebrates are homologous with the varied types of 'Golgi apparatus' in other cells is still a matter of controversy among cytologists (Baker, 1954; Cain, 1954); but the account of the dictyosomes in the neurones of *Patella* as seen in electron microscope sections (Lacy, 1957) agrees very well with the fine structure of the 'Golgi apparatus' in many other types of cells (compare Gatenby and Lutfy, 1956; Pollister and Pollister, 1957).

The secretory product of the neurone is the axon. There is now good evidence for a continuous secretion of axoplasm by the cell-body of the neurone (Koenig, 1958; Weiss, 1959). The large axons of the cockroach have been shown in the present paper to be highly organized at the light microscope level and made up of a great number of neurofibrils. It was therefore reasonable to expect some organized structure within the cell-body responsible for the secretion of these fibrils of axoplasm.

The histological evidence here presented suggests that the neurofibrils are traceable to the dictyosomes. There are all intermediate stages between cuffs of osmiophil material around unstained neurofibrils as they enter the base of the axon, and typical Golgi bodies of annular or horse-shoe form, the unstained core of which is continuous with unstained filaments that are thought to be neurofibrils. The orientation of the dictyosomes, with their unstained core or long axis concentric with the nuclear membrane in the body of the cell, and converging upon the base of the axon when they lie in the axon cone, reinforces this impression.

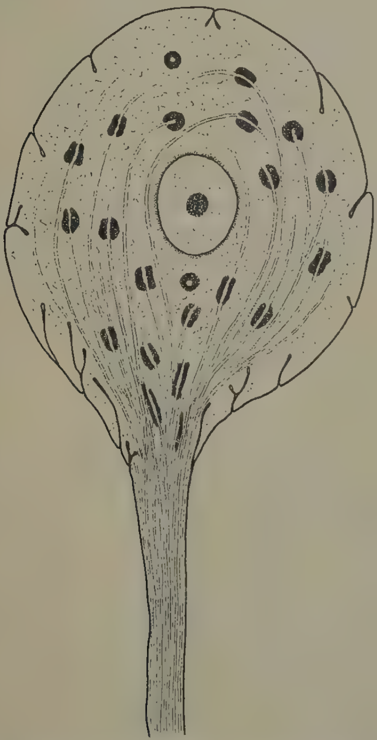

FIG. 4. Schematic drawing of ganglion cell showing glial invaginations and the suggested relation between dictyosomes and neurofibrils.

Fig. 4 is a schematic illustration of the relation that is inferred. Golgi bodies or a series of Golgi bodies are pictured as providing the matrix for individual neurofibrils. No method is available which will stain both axoplasm and Golgi bodies in the same preparation, and it is impossible to follow unstained filaments very far in the granular cytoplasm of the ganglion cells. It has therefore not been possible to make preparations which show irrefutably that every Golgi body is connected to a neurofibril, but that is what the evidence suggests.

It must be emphasized that these unstained strands which give a rope-like appearance to the large axons are structures of half a micron or so in thickness. They are quite distinct from the long slender filaments, 50–100 Å in diameter, which are so conspicuous in thin sections of axons examined with the electron microscope (Fernández-Morán, 1952; Vial, 1958; Wigglesworth, 1959*b*). These filaments of fibrous protein form less than a tenth of 1% of the total axon volume (Schmitt, 1957), whereas the unstained neurofibrils here described (0·25 to 0·5 μ in diameter) make up the bulk of the axon as seen with the light microscope, and there is relatively little stainable material between.

It is this unstained strand, which must contain a large number of the widely spaced 100-Å filaments, that has been referred to throughout this paper as a neurofibril. In their studies on the structure of the mammalian neurone Palay and Palade (1955) showed more or less parallel arrays of the 100-Å filaments running in clear channels, some half a micron or less in width, between the Nissl bodies. It is channels of this type which would correspond with the neurofibrils discussed in this paper.

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The Electron Microscopy of the 'Golgi Apparatus' in the Purkinje Cells of Owls

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With four plates (figs. 1–4)

SUMMARY

The classical site of the 'Golgi apparatus', the Purkinje cells of the cerebellum of owls, has been examined by electron microscopy. The greater part of the cytoplasm consists of aggregates of closely packed granular membranes of endoplasmic reticulum. The objects described by Dalton and Felix in electron micrographs and called by them the Golgi apparatus are rarely seen in these preparations. It seems likely that the 'Golgi apparatus' of this cell as seen in the optical microscope is formed by the deposition of silver or osmium on the membranes of the endoplasmic reticulum, which form a network throughout the cytoplasm of this cell.

INTRODUCTION

IT was shown by one of us (Malhotra, 1959*a*) that the classical 'Golgi apparatus' of the perikaryon of the neurone of vertebrates was a deposit of silver or of osmium on a reticulum, which could be seen in the living cell by interference microscopy. This reticulum was identified as the basiphil reticulum or *Netz*, described by Nissl (1894), in these cells. These conclusions were also supported by the study of the Golgi apparatus in its classical site, that is to say, the Purkinje cells of the cerebellum of owls (Malhotra, 1960). The evidence suggested that the bulk of the 'Golgi apparatus' of these cells was a deposit of silver or of osmium on the endoplasmic reticulum, with which small basiphil granules are associated (Palade, 1955*a*, 1958; Palay, 1956, 1958; Palay and Palade, 1955).

It has become customary among electron microscopists (Dalton and Felix, 1954; Sjöstrand, 1956; Lacy and Challice, 1957; Pollister and Pollister, 1957; Palay, 1958) to describe as Golgi apparatus in various cells a system of vacuoles, small vesicles, and closely packed parallel membranes, characteristically devoid of the ribonucleoprotein granules of Palade (1955*b*, 1958) that are found in association with the granular endoplasmic reticulum. This implies that the Golgi 'apparatus' or 'network', as originally described by Golgi and as seen in typical Golgi preparations under the light microscope, is always composed of such elements. The purpose of this investigation was to find out what structures seen in the electron microscope might correspond with the typical network seen in the classical site described by Golgi himself in his original publication (Golgi, 1898), namely, the Purkinje cells of the owl.

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MATERIAL AND METHODS

Living owls are very difficult to obtain. We were fortunate at the beginning of this investigation in obtaining from Mr. Robert Jackson of Hale, Cheshire, two live tawny owls, *Strix aluco*. The greater part of the cerebellar tissue from these two birds was used in making vital observations, in following Golgi's original method of silver impregnation, and in carrying out histochemical studies. Very little was available for electron microscopy. The small amount of material from one bird was fixed in Palade's osmium tetroxide fixative and embedded in methacrylate. The preservation of the Purkinje cells in these preparations was considered poor by modern standards, and a search was made for more tawny owls. These proved to be unobtainable, but one live spotted owl, *Bubo africanus*, was obtained from the Regent Pet Stores, Camden Town, London. The whole of the cerebellum of this bird was used to prepare more material for electron microscopy. Fixations were made in both the standard Palade medium and in buffered potassium permanganate; araldite was used for embedding.

Strix aluco. The owl was killed by chloroform, and the cerebellum was dissected out immediately after death. Pieces of tissue, about 1-mm cubes, were cut out, fixed in ice-cold 1% buffered osmium tetroxide solution at pH 7·2 for 2 h (Palade, 1952), dehydrated in graded alcohols, and embedded in *n*-butyl methacrylate.

Bubo africanus. The owl was lightly anaesthetized with ether and the cerebellum quickly removed in two pieces. One piece was placed in Palade's fixative and one in 1% potassium permanganate solution buffered at pH 7·3 by veronal/acetate (Luft, 1956). Each piece was at once cut into 1-mm cubes with a sharp razor-blade. Material was fixed in Palade's medium for ½, 1, and 2 h; in Luft's medium for 15 min, ½, and 1 h. The fixative was kept ice-cold in each case. The tissue blocks were then dehydrated in graded alcohols and embedded in araldite (Glauert and Glauert, 1958).

Blocks were in all cases sectioned on a Porter–Blum microtome. Methacrylate sections were mounted on carbon-filmed Smethurst Highlight grids; araldite sections were examined on unfilmed grids. Only sections showing silver or grey interference colours were used. The sections were examined in a modified Siemens Elmiskop I (Meek, 1960), with the double condenser (400-μ aperture), a beam current of 10-μA, and an objective aperture of 30-μ diameter.

RESULTS

The electron micrographs of the Purkinje cells of the first preparation of the cerebellum of *S. aluco* showed very widely dilated cisternae of the endoplasmic reticulum. These apparent spaces were filled with featureless

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FIG. 1 (plate). Electron micrograph of a Purkinje cell of *B. africanus* (from tissue fixed in Palade's fluid and embedded in araldite). *er*, endoplasmic reticulum; *l*, lipid globules; *n*, nucleus.

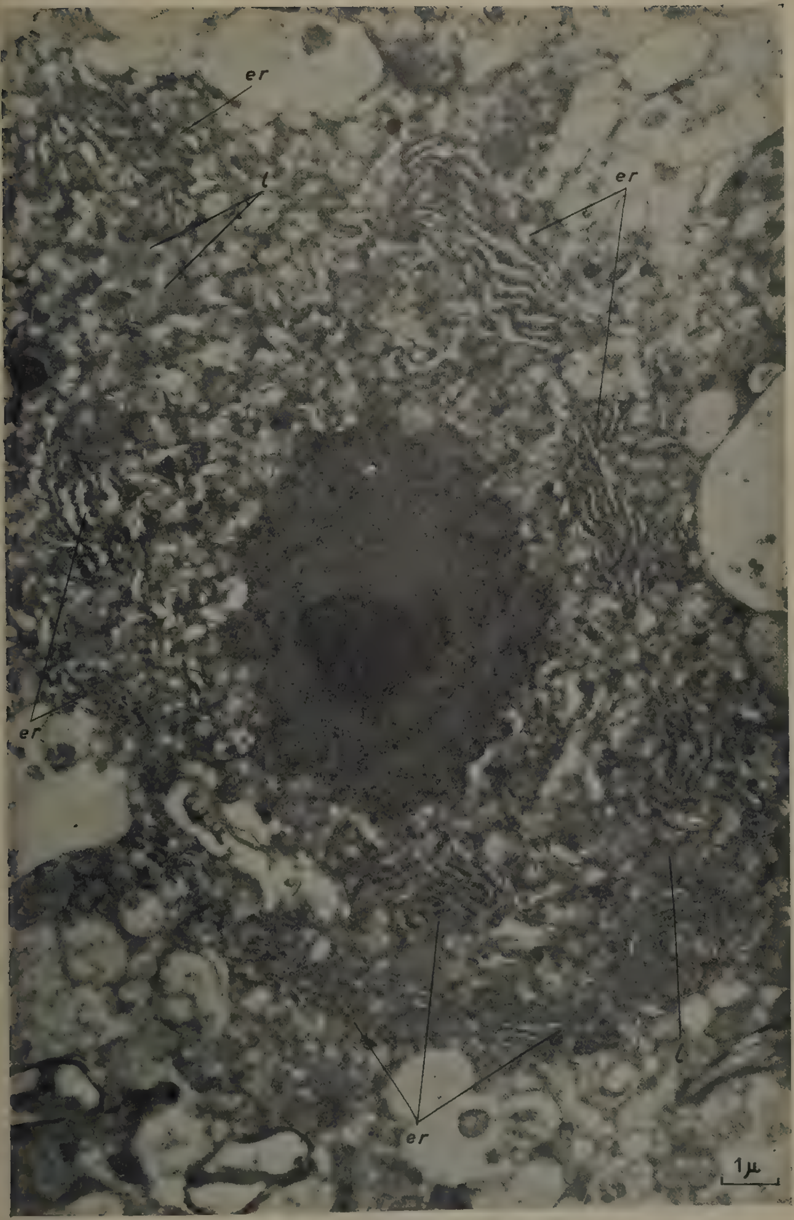

FIG. 1

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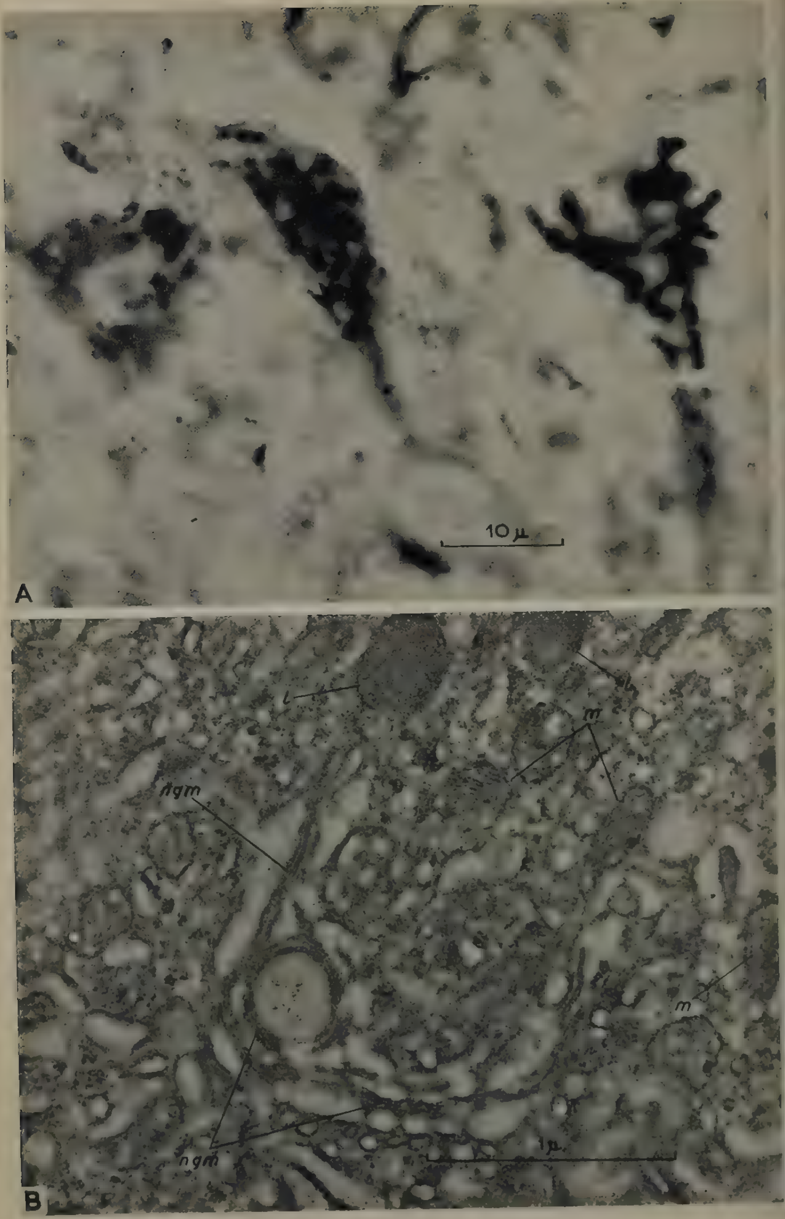

FIG. 2

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embedding material. It was thought that these spaces might be artifacts of fixation or embedding. Pieces of the cerebellum of *B. africanus* were prepared with the greatest care and embedded in araldite. The cisternae of the endoplasmic reticulum of the Purkinje cells of this owl were seen to be almost as widely dilated as in the first preparation. It was therefore concluded that these spaces were probably not artificial. No significant difference could be seen between the cytoplasmic inclusions of the Purkinje cells of the two species of owl, and the following description applies equally to both.

Four kinds of cytoplasmic inclusions can be clearly recognized in the electron micrographs. These are:

(1) endoplasmic reticulum with associated particles of Palade (1955*a*, 1958);
(2) non-granular membranes or γ-cytomembranes associated with vacuolar structures (the 'agranular reticulum' of Palay and Palade, 1955) often described as the 'Golgi apparatus' (Dalton and Felix, 1954; Hess, 1955; Sjöstrand, 1956; Lacy and Challice, 1957; Pollister and Pollister, 1957; Palay, 1958; Oberling, 1959);
(3) rounded bodies, presumably sections of spherical or ovoid lipid inclusions;
(4) mitochondria.

Endoplasmic reticulum. The most obvious and extensive structure seen in the cytoplasm is the granular endoplasmic reticulum. The elements of this system mostly form aggregates of varying sizes (fig. 1). These correspond to the Nissl bodies of preparations stained for light microscopy. The membrane-bounded cisternae of this reticulum are arranged in a more orderly way than in the Purkinje cells of other vertebrates (compare Palay and Palade, 1955). Very often they are placed more or less in parallel rows like those seen in the dorsal root ganglion cells (Hess, 1955; Palay and Palade, 1955). When this is so, the rows are more or less equidistant. A considerable variation in the width (reaching up to about 100 mμ) of the cavities of the endoplasmic reticulum is seen in the neurones fixed in buffered osmium tetroxide solution embedded in methacrylate (fig. 3) or araldite (fig. 4, A). Palay and Palade (1955) give 30–50 mμ as the common figures for the diameter of this lumen in the neurones of the rat. It is difficult to be certain whether the cisternae of the endoplasmic reticulum of the Purkinje cells of the owl are very wide in life. Living neurones of vertebrates show certain 'canalicular spaces' of very low refractive index in the cytoplasm; these are bigger in the Purkinje cells of the owl (Malhotra, 1960) than in the neurones of the mouse (Malhotra,

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FIG. 2 (plate). A, light micrograph of an Aoyama preparation, showing 3 Purkinje cells of *S. aluco* with reticular apparatus of Golgi.

B, electron micrograph of part of the cytoplasm of a Purkinje cell of *S. aluco* (from material fixed in Palade's fluid and embedded in methacrylate). *l*, lipid globules; *m*, mitochondria; *ngm*, non-granular membranes.

1959*a*). These canalicular spaces may be the dilated cisternae of the endoplasmic reticulum seen in electron micrographs.

A very different appearance is presented by the cytoplasm of the Purkinje cells fixed in buffered potassium permanganate solution. The cisternae of the endoplasmic reticulum have disappeared, leaving well-preserved, closely apposed pairs of membranes (fig. 4, B). The rosettes of granules which are so prominent in the ground cytoplasm surrounding the cisternae in the osmium-fixed cytoplasm (fig. 4, A) are no longer present in the permanganate-fixed material. This is in accordance with the findings of Bradbury and Meek (1960) in exocrine pancreas cells. In view of the fact that permanganate has been shown to remove many of the cell components, it is probable that fixation by buffered osmium tetroxide gives a closer representation of the structures found in the living cell. It may be mentioned that the nuclear membrane is particularly prominent after permanganate fixation, and shows well-defined pores (fig. 4, B).

Occasionally, within an aggregate of the endoplasmic reticulum, an appearance resembling non-granular membranes is seen (fig. 3, A; arrows). The general morphology of these structures differs from the typical non-granular membrane system (part of the Golgi apparatus of Dalton and Felix, 1954), which is very rarely seen in the electron micrographs of this cell (fig. 2, B; *ngm*). In the typical non-granular membrane system, many membranes are closely packed against one another. In fig. 3, A the arrows show only two membranes in apposition, and these appear to be the membranes of two different cisternae. The space between them is very small, and there is scarcely room for the small granules of Palade. Thus the two membranes in apposition give, here and there, the appearance of 'non-granular membranes'. If they are indeed non-granular, they provide the best illustration yet of the continuity of granular and non-granular endoplasmic reticulum. However, close scrutiny of the narrow space between these membranes occasionally reveals particles of about the same size as Palade's granules (fig. 3, B, C). This appearance may, therefore, have been caused by artificial swelling of the cisternae of the endoplasmic reticulum resulting in a close apposition of the granular membranes. We are inclined to believe that this is the correct interpretation.

Elements of the endoplasmic reticulum are also scattered in the cytoplasm between the highly organized aggregates. They presumably correspond to the basiphil strands seen in Nissl preparations, connecting the aggregates into a reticulum. The aggregates of endoplasmic reticulum are seen in the dendrites of the cell, but not in the axons.

FIG. 3 (plate). A, electron micrograph of a typical aggregate of endoplasmic reticulum. *S. aluco*; fixed in Palade's fluid and embedded in methacrylate. Arrows indicate closely apposed membranes continuous with the membranes of the endoplasmic reticulum.

B, C, electron micrographs of *S. aluco* (Palade/methacrylate) at higher magnification than A, showing particles of Palade (*g*) in the ground cytoplasm between one cisterna (*c*) and another. In some places the cisternae are closely apposed to one another, leaving only a small intervening space in which the particles are visible here and there.

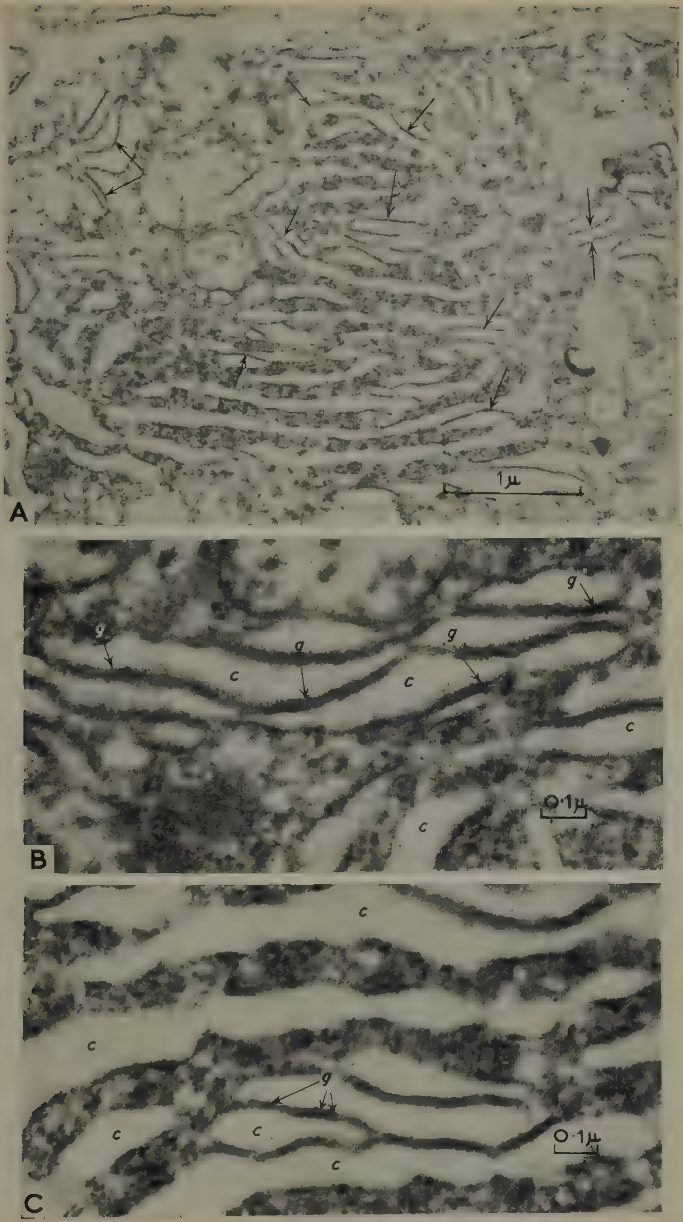

Fig. 3

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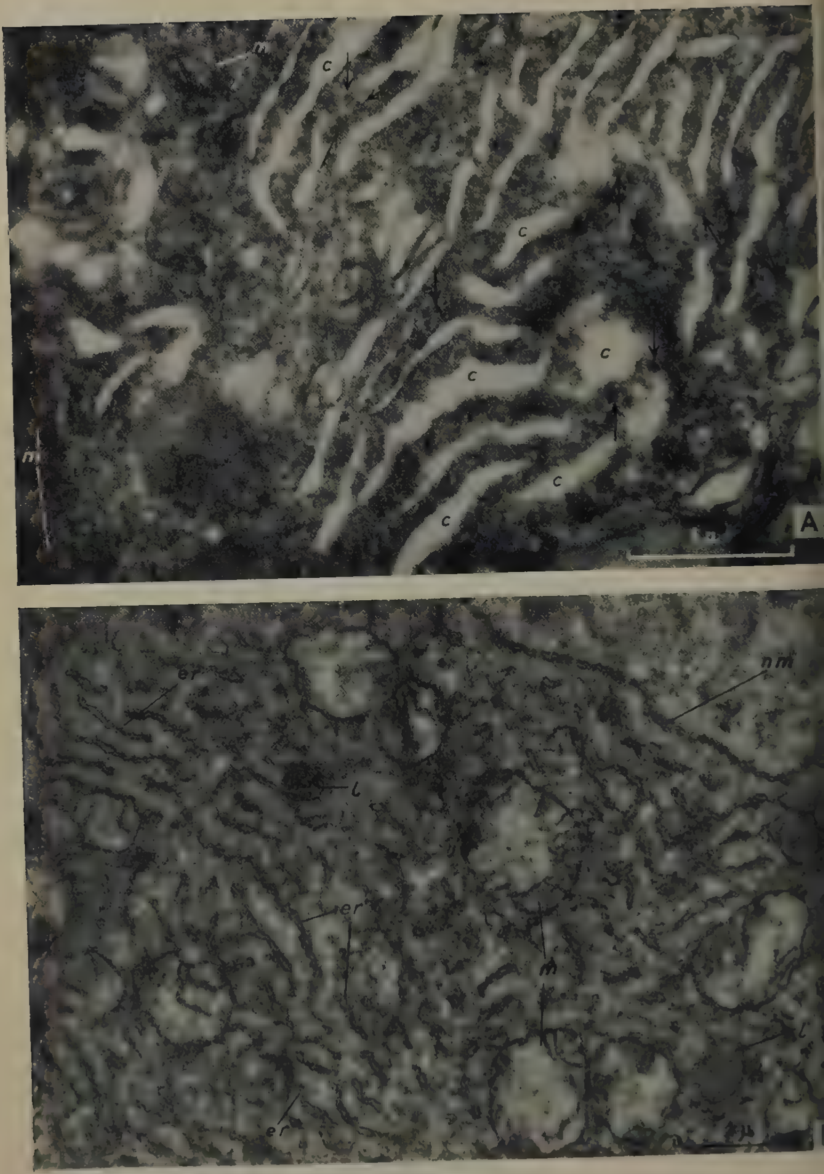

FIG. 4

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Non-granular membranes. The second structure is very rarely seen in the electron micrographs (fig. 2, B). It is composed of non-granular membranes associated with round or elongate vacuoles of very low electron density. These membranes are more closely packed than the elements of the granular endoplasmic reticulum. They are very scanty in the electron micrographs of the Purkinje cells of the owls.

Rounded bodies regarded as lipid globules. Most of the spherical or almost spherical bodies seen in the micrographs are bounded by a thin membrane (figs. 1; 2, B; 4, B). These bodies have a finely granular appearance and are electron-dense: the largest is a little less than 1 μ in diameter. Some of these inclusions show vacuoles of lower electron-density in them.

Mitochondria. These show an outer limiting membrane and the characteristic cristae (figs. 2, B; 4) (Palade, 1953; Hess, 1955; Palay and Palade, 1955; Andrew, 1956; Sjöstrand, 1956). They are spherical or elongate, narrow bodies. The longest seen in the electron micrographs measure about 3 μ.

DISCUSSION

The general morphology of the endoplasmic reticulum seen in the electron micrographs of the Purkinje cells of *S. aluco* and *B. africanus* corresponds with the Golgi preparations of this cell (see fig. 2, A). It also resembles the illustration of the 'reticular apparatus' of Golgi (1898) in the Purkinje cells of *S. flammea.* It therefore seems likely that the Golgi apparatus seen in this cell by light microscopy is a deposit of silver or of osmium or of compounds of these metals on the endoplasmic reticulum. This finding is in accordance with the conclusions already reached by one of us (Malhotra, 1959*a*, 1960). The elements of the non-granular membranous system seen in the electron micrographs of the Purkinje cells of the owl are not nearly abundant enough to form the massive apparatus of Golgi. In view of these conclusions the use of the term 'Golgi apparatus' by Hess (1955), Palay (1958), and Oberling (1959) for the non-granular membranes of the neurones of vertebrates does not seem to be appropriate.

It has previously been shown that a reticulum, which corresponds in distribution to the endoplasmic reticulum, can be seen in the living Purkinje cell of the owl (Malhotra, 1960). This reticulum has an unusually great affinity for silver. The evidence suggested either that the endoplasmic reticulum of this particular cell contained more phospholipid than usual, or that its phospholipid component was more easily unmasked.

FIG. 4 (plate). A, electron micrograph of a typical aggregate of endoplasmic reticulum. *B. africanus*; Palade/araldite; compare with fig. 3. The arrows indicate rosettes of particles of Palade. *c*, cisternae; *m*, mitochondria.

B, electron micrograph of part of a Purkinje cell of *B. africanus*. Buffered potassium permanganate / araldite. Note the high contrast of the membrane structures of the endoplasmic reticulum (*er*), mitochondria (*m*), and nuclear membrane (*nm*) with well-defined pores. The membranes of the endoplasmic reticulum are closely apposed and not separated by cisternae as in material fixed in buffered osmium tetroxide (fig. 4, A). *l*, lipid globules.

It was stated earlier (Malhotra, 1959*a*) that the endoplasmic reticulum is not by any means always blackened by the Golgi methods. In the exocrine cell of the pancreas, where it exists in its closely packed form (Palade, 1955*a*, 1958; Sjöstrand, 1956; Haguenau, 1958), it was known as 'ergastoplasm' long before the use of the electron microscope in biology. The 'ergastoplasm' of the pancreatic cell and the 'Nissl substance' of the neurone of vertebrates are similar in their ultrastructure, but they react very differently to acid dyes. The ergastoplasm (besides being intensely basiphil) is also remarkably acidophil (Baker, 1959), whereas the Nissl substance shows no affinity for acid dyes (Malhotra, 1959*b*). In view of this striking difference in response to dyes, it is perhaps not surprising that the two objects react differently to osmium tetroxide and silver nitrate.

It is a pleasure to thank Dr. J. R. Baker, F.R.S., and Dr. R. Barer for their valuable criticism throughout the course of this work. This work was made possible by the generous loan of a Siemens electron microscope by the Wellcome Trust to the Department of Human Anatomy, Oxford. One of us (S. K. M.) is a Postdoctoral Research Fellow of the Panjab University (India), and received a Travel Grant under the Commonwealth University Interchange Scheme of the British Council.

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The Chromosome Numbers and Nuclear Cytology of some Common British Serpulids

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With two plates (figs. 1 and 2)

SUMMARY

The chromosome numbers of *Hydroides norvegica*, *Mercierella enigmatica*, and *Pomatoceros triqueter* were determined from squashes of somatic cells in young embryos obtained by artificial fertilization, and stained with iron-alum/aceto-carmine. All had a diploid count of $2n = 26$ chromosomes. Mitotic and meiotic divisions in the 5 species of *Spirorbis* examined, and in *Filograna implexa*, all revealed a diploid chromosome number of $2n = 20$. A diploid chromosome number of 14 is suggested for the ancestral serpulid.

INTRODUCTION

INVESTIGATIONS dealing with the nuclear cytology and chromosome numbers in the polychaete worms have been neglected in the past. This group has both hermaphrodite and bisexual forms and includes a wealth of species with diverse modes of life. It seemed that investigations of chromosome number and nuclear cytology in Polychaeta, along similar lines to the work on lumbricids (Muldal, 1949), might yield results of taxonomic value and indicate probable lines along which the evolution of the group has taken place.

While chromosome numbers vary considerably in different families of Polychaeta, there may also be diverse counts within one family. *Amphitrite* sp. has a diploid count of $2n = 22$ (Scott, 1906), while *Lanice conchilega* Malgreu, which belongs to the same family, the Terebellidae, has a count of $2n = 6$ (Dehorne, 1911). Polyploidy may have played a part in the evolution of the group; the phenomenon seems widespread in hermaphrodite annelids (White, 1940). On the whole, however, higher counts seem predominant among sedentary polychaetes, e.g. *Aricia* sp. with $2n = 18$ (Kostanecki, 1909); *Chaetopterus pergamentaceus* Cuvier with $2n = 18$ (Mead, 1898), and *Serpula crater* Claparède with $2n = 14$ (Soulier, 1906).

METHODS

Mitotic and meiotic divisions were studied from preparations made by an iron-alum/aceto-carmine squash technique (Belling, 1926) involving the use of a separate bath of iron-alum mordant (Godward, 1948; Austin, 1959).

In the sub-family Serpulinae, the preparations were made from developing embryos obtained by means of artificial fertilization. Male and female worms

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were taken out of their tubes and placed in a dish containing sea-water. The sex cells were seen streaming out of the body and were allowed to mix together for fertilization to take place. Føyn and Gjøen (1954) described artificial fertilization in *P. triqueter* L. and we found it to be equally successful in *H. norvegica* and *M. enigmatica*. At 18° to 20° C the first cleavage in these 3 species occurred between $1\frac{1}{2}$ and $1\frac{3}{4}$ h after fertilization. The best preparations were obtained with embryos between 2 and 4 h old, when active cell-division was taking place to form a many-celled blastula. Later stages were less suitable as the rate of mitosis slowed down. Cell-division almost stopped after the trochophore stage.

In the sub-family Spirorbinae both embryos and mature worms were examined. *Spirorbis borealis* shows a tidal periodicity in liberation of its larvae (Garbarini, 1933). They are liberated during neap tides, so that the ideal time for collection of material both for maturation division in adult worms and for mitotic activity in the embryos occurs a few days before spring tides, i.e. between the last batch of liberated larvae and the next spring tide.

In *F. implexa* mitotic divisions in somatic cells of young worms were examined. Table 1 (see Appendix, p. 400) gives the sources of material of the species studied.

Observations

The cytological observations made on the species under study are summarized in table 2 (see appendix). Both in mitosis and in meiosis the prometaphase and metaphase presented the clearest figures from which counts could be made.

The species *H. norvegica* (Gunnerus), *P. triqueter* L., and *M. enigmatica* Fauvel were found to possess a chromosome number of $2n = 26$. The centromere occurs in a terminal or sub-median position on the chromosomes of all three species (fig. 1, A, B, D, E). The chromosomes are all of similar size and form. At metaphase the chromosomes arrange themselves around a central spindle element (fig. 1, B) and anaphase separation is normal (fig. 1, C). The only available material of *Serpula crater* Claparède yielded only a single countable nucleus; this confirmed Soulier's count of $2n = 14$.

In the Spirorbinae, 5 species of *Spirorbis* were investigated. Both mitotic divisions in somatic cells and meiotic divisions during spermatogenesis were studied. All 5 species showed a uniform count of $2n = 20$ (fig. 2, A–F). In general, the chromosomes of this sub-family have terminal and sub-terminal centromeres. At meiotic metaphase I, 10 bivalents are discernible which assume characteristic shapes, there being no central element in the spindle.

FIG. 1 (plate). A, early mitotic prophase of *H. norvegica*: chromosomes in extended state.

B, mitotic metaphase of *H. norvegica*, showing the 26 chromosomes in one plane. Central elements are present in the spindle.

C, late mitotic anaphase in *H. norvegica*.

D, mitotic prophase in *M. enigmatica*: the 26 chromosomes can be counted.

E, late mitotic prophase or prometaphase in *P. triqueter*: the 26 chromosomes can be counted.

F, polar view of metaphase plate of *F. implexa*.

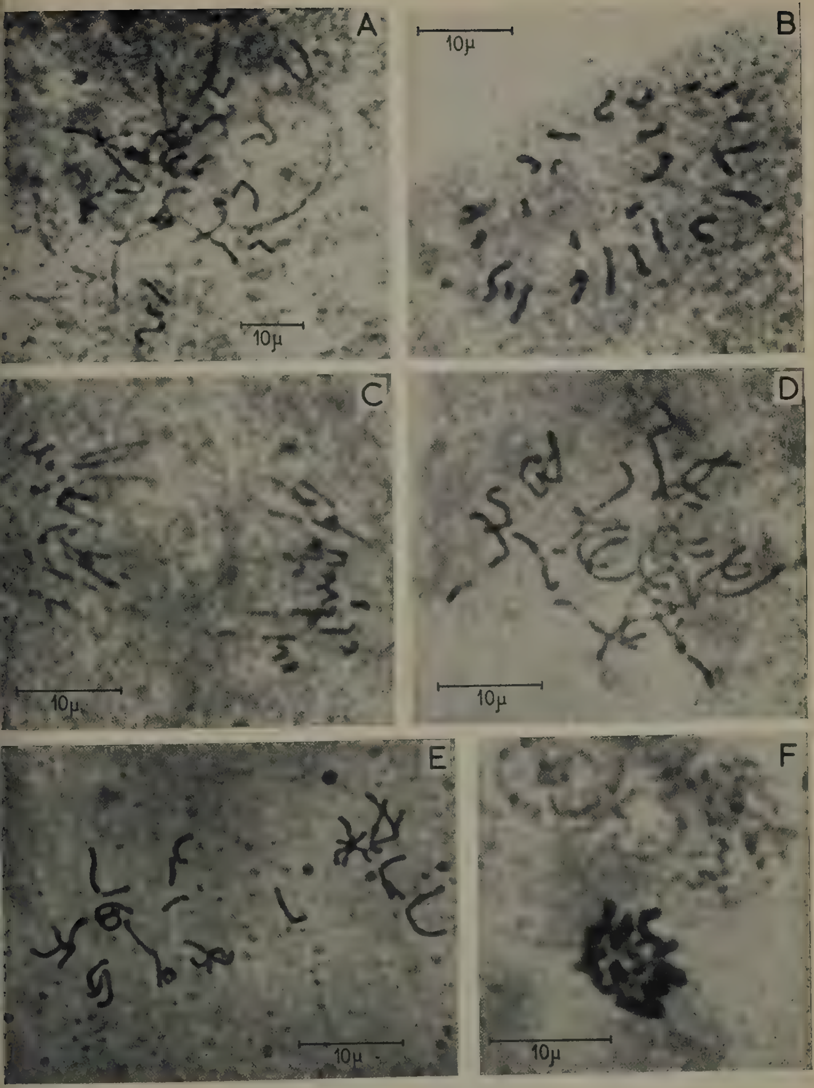

FIG. 1

S. DASGUPTA *and* A. P. AUSTIN

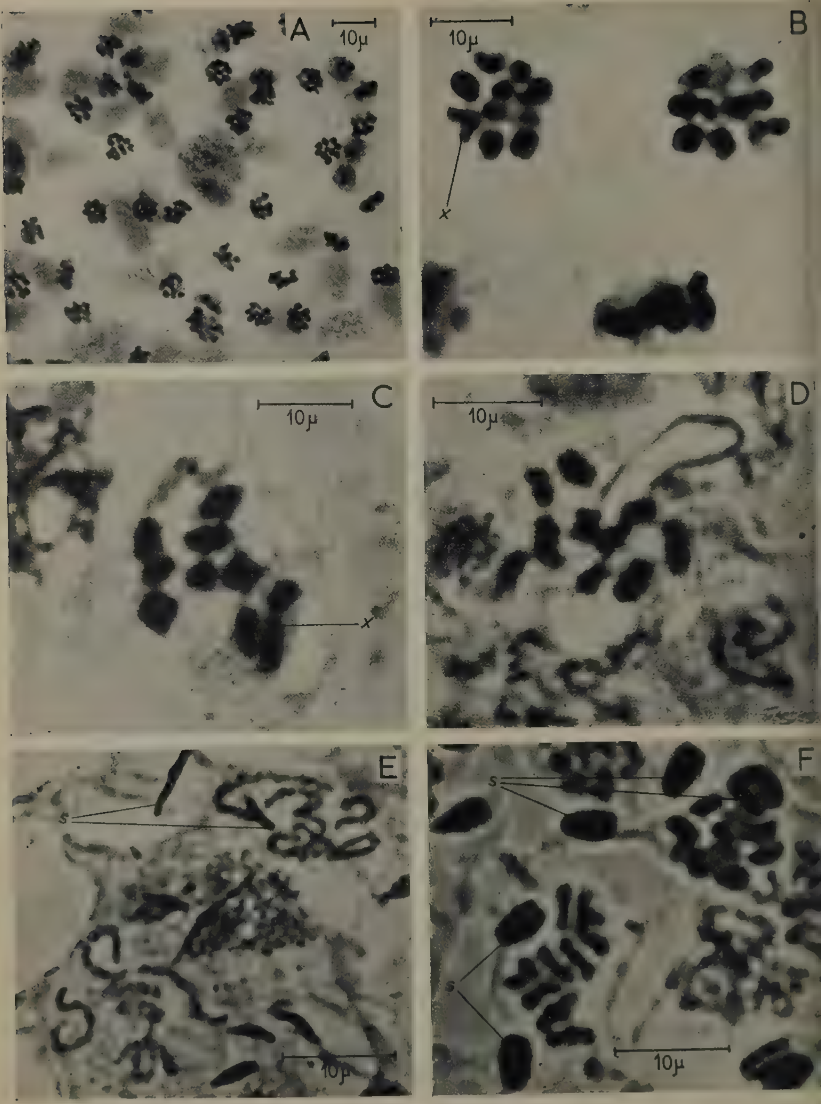

FIG. 2

S. DASGUPTA *and* A. P. AUSTIN

A possible hybrid between *S. borealis* and *S. corallinae* has been reported by de Silva (1960).

At metaphase II the chromosomes are highly contracted and dot-like. In *S. borealis* (fig. 2, B) and *S. corallinae* (fig. 2, C) one of the chromosomes (marked with an *X*) is larger than the rest, while in the other species they are all of about the same size (fig. 2, D–F). The nucleolus could be demonstrated only in *S. tridentatus*. It disappeared in early prophase (fig. 2, E).

In *F. implexa* (figs. 1, F; 3) the chromosome number is 20. Ten distinct bivalents can be seen during meiosis in both spermatogenesis and oogenesis. Chromosome size and position of centromere are similar to those found in the Spirorbinae.

DISCUSSION

The family Serpulidae has three sub-families: the Serpulinae, Spirorbinae, and Filograninae. While the Spirorbinae and Filograninae are hermaphrodites, *P. triqueter* (Føyn and Gjøen, 1954) and *H. norvegica* (Ranzoli, 1954) of the Serpulinae are protandrous hermaphrodites. The chromosome numbers in this family are as follows:

(*a*) Serpulinae	
H. norvegica (Gunnerus)	$2n = 26$
M. enigmatica Fauvel	$2n = 26$
P. triqueter L.	$2n = 26$
Serpula crater Claparède	$2n = 14$
(*b*) Spirorbinae	
Spirorbis borealis Daudin	$2n = 20$
S. corallinae de Silva (1960)	$2n = 20$
S. tridentatus Levinsen	$2n = 20$
S. pagenstecheri Quatrefages	$2n = 20$
S. spirillum L.	$2n = 20$
(*c*) Filograninae	
F. implexa (Berkeley)	$2n = 20$

The occurrence of $2n = 14$ in *Serpula crater* and $2n = 26$ in the other three species suggests that polyploidy may have played a part in the evolution of some of the species of the Serpulinae (fig. 4). If in this way the original

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FIG. 2 (plate). A, synchronous division (meiotic metaphase II) of groups of nuclei in testes of *Spirorbis borealis*.

B, meiotic metaphase II in testes of *S. borealis*: 10 bivalents can be counted, including one dot and one large chromosome (marked *X*).

C, D, E, meiotic metaphase I of *S. corallinae*, *S. pagenstecheri*, and *S. tridentatus*, to show 10 bivalents in each case. In *S. corallinae* (C) the large chromosome is marked *X*. Many sperm heads can be seen in E. *s*, sperm heads.

F, meiotic prophase I of *S. spirillum*. The chromosomes are extended and the developing sperm can be seen. *s*, sperm heads.

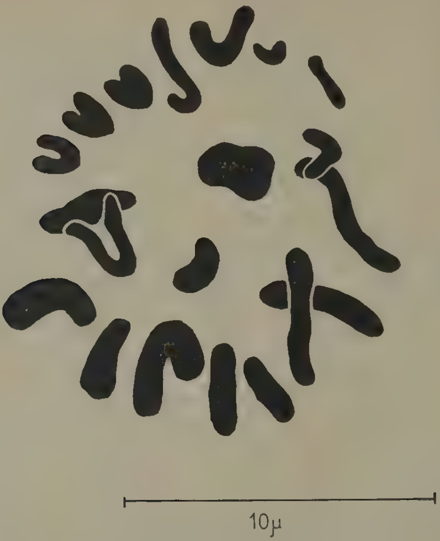

Fig. 3. Mitotic metaphase in *F. implexa*, showing 20 chromosomes of considerably differing sizes.

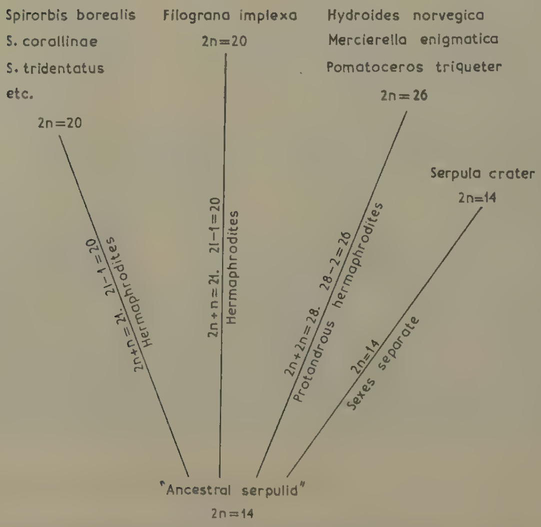

Fig. 4. Diagram showing the possible mode of evolution of chromosome number in the family Serpulidae.

number $2n = 14$ was doubled to $2n = 28$, then, by the loss of a pair of chromosomes in the course of evolution, the count found in *Hydroides*, *Mercierella*, and *Pomatoceros* of $2n = 26$ could have been arrived at. If we take a form with $2n = 14$ as the 'ancestral serpulid', the families Filograninae and Spirorbinae could be considered as $2n+n-1 = 20$, with the loss of a single chromosome taking place in the course of evolution of the group. The assumption that one chromosome from the haploid constituent is lost is supported in the sub-group Serpulinae, where two may have been lost (see fig. 4).

We are grateful to Dr. D. J. Crisp for his ready encouragement, to Dr. C. Burdon-Jones for supplying specimens of *F. implexa*, and to Dr. T. B. Reynoldson and Dr. D. H. Stone for valuable criticism.

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APPENDIX

TABLE 1. *Sources of material*

Species	*Breeding season*	*Usual habitat*	*Collecting-ground*
H. norvegica (Gunnerus)	Aug.	Stones and shells	Swansea, Queen's Dock
P. triqueter L.	Mar.–Apr.	Stones, rock	Menai Straits
M. enigmatica Fauvel	Summer	Stones, rock	Swansea Dock and Milford Dock
Spirorbis borealis Daudin	Apr.–Nov.	*Fucus serratus*	Menai Straits
S. corallinae de Silva (1960)	Summer	*Corallina officinalis*	Menai Straits and Rhosneigr
S. tridentatus Levinsen	Summer	Pebbles and stones	Menai Straits and Holyhead harbour
S. pagenstecheri Quatrefages	Summer	Boulders and rock	Menai Straits and Holyhead harbour
S. spirillum L.	Summer	Bases of hydroids	Dredged off Puffin Island
F. implexa Berkeley	July, Aug., Sept.	Stones and shells	Dredged off eastern Anglesey

TABLE 2. *Cytological details of the nuclei of members of the Serpulinae, Spirorbinae, and Filograninae investigated*

Species	*Haploid chromosome number (n)*	*Diploid chromosome number (2n)*	*Diameter (in μ) of resting nucleus*	*Diameter of nucleolus (in μ)*	*Prophase Average size in μ*			*Prometaphase Average size in μ*		
					Nucleus diameter	*Chromosome bivalent, length*		*Nucleus diameter*	*Chromosome bivalent, length*	
						largest	*smallest*		*largest*	*smallest*
Serpulinae:										
H. norvegica	13	26	15·6	. .	. .	25·0	6·0	. .	9·5	3·0
M. enigmatica	13	26	16·7	. .	. .	. .	. .	14·25	. .	. .
P. triqueter	13	26	. .	. .	. .	. .	. .	13·5	. .	. .
Spirorbinae:										
S. borealis	10	20	15·15	. .	16·5	13·5	4·12	14·85	7·25	3·0
S. corallinae	10	20	18·15	. .	22·5	15·0	4·5	20·62	7·5	3·0
S. tridentatus	10	20	12·0	2·25	14·7	12·5	4·5	12·6	6·0	2·0
S. pagenstecheri	10	20	9·6	. .	14·1	13·5	4·5	. .	7·5	3·0
S. spirillum	10	20	9·75	. .	13·2	14·25	3·0	. .	. .	. .
Filograninae:										
F. implexa	10	20	14·10	. .	. .	. .	13·5	. .	. .	. .

Development of the Testis Tubule in the Fowl

By GAMAL A. R. KAMAR

(*From the Animal Production Department, Faculty of Agriculture, Cairo University, Giza, Egypt*)

With three plates (figs. 1–3)

SUMMARY

Three phases of tubule development can be distinguished in males of the Fayomi breed:

(i) the first 12 weeks, when only slight increase in tubule diameter and cell count occurs. During this period two stages in spermatogenesis are to be found.

(ii) from the 16th to the 24th week, when rapid increase in tubule diameter and cell count occurs, and all stages of spermatogenesis are present. Sexual maturity, judged by the appearance of the first spermatozoa in the seminiferous tubules, occurs at about 16 weeks and is associated with a rapid increase in the size of the testes. The increase in testis weight is due, however, to increase in length of the tubules rather than to increase in diameter.

(iii) from the 24th to the 52nd week, when only slight changes occur in microscopical appearance.

INTRODUCTION

IN normal male fowls, four developmental stages of spermatogenetic activity have been observed (Hiatt and Fisher, 1947; Kumaran and Turner, 1949; Charny, Conston, and Meranze, 1952). The first occurs during the first 5 weeks, when the tubules are organized and gradually increase in diameter. The spermatogonia also multiply during this first period. In the second phase, primary spermatocytes begin to appear in the sixth week, and during the next 2 to 3 weeks the growth of the layer of primary spermatocytes takes precedence over further multiplication of the spermatogonial layer. In the third phase, secondary spermatocytes begin to appear at about 10 weeks. During the fourth stage, spermatids appear in the tubules of birds 12 weeks old. By the twentieth week, spermatids are usually present in all the seminiferous tubules. During this period, there is also marked growth in length of the tubules, and thus the capacity of the testes to produce spermatozoa is increased (Kumaran and Turner, 1949).

Sexual maturity is reached with the appearance of the first spermatozoa in the seminiferous tubules, at about 16 weeks. The age at which this occurs varies according to breed, locality, management, and nutrition. In pen-matings, however, a satisfactory level of fertility may not be reached until the cockerels are 26 weeks old (Hogue and Schnetzler, 1937; Parker, McKenzie, and Kempster, 1942).

The present study was designed to follow in detail the process of tubule development in males of the Fayomi breed.

[Quarterly Journal of Microscopical Science, Vol. 101, part 4, pp. 401–6, 1960.]

MATERIAL AND METHODS

Thirty-nine Fayomi males were killed at ages ranging from 4 to 52 weeks at intervals of 4 weeks. Up to the time of killing they received normal, balanced rations, and rearing and management were standard. Groups of these birds were weighed alive and then killed in groups of 3 at the following ages and on the following dates:

Age in weeks	*Date of killing*	*Age in weeks*	*Date of killing*
4	2 December	32	21 July
8	28 December	36	18 August
12	25 January	40	15 September
16	22 February	44	18 October
20	29 March	48	15 November
24	24 April	52	14 December
28	1 June		

The testes were dissected out and weighed immediately on a torsion balance. The average weight of the pair of testes per bird at each age (given in the Appendix, p. 405) was calculated by dividing the total weight of right and left testes from all three males at each age by three.

Samples were taken from the middle of each of the two testes of the three individuals in each age-group. The samples were fixed in Bouin's fluid, washed, dehydrated, cleared, embedded in paraffin wax, sectioned at 10 μ, stained with haematoxylin and eosin, and mounted in Canada balsam. Sections from each age-group were examined microscopically, and the different stages of spermatogenetic activity were noted. Microscopical examination was carried out with a projector microscope. The number of seminiferous tubules in each projector field was counted, and the number per square cm of section was calculated. The diameters of the seminiferous tubules were measured in microns. For this purpose tubules were usually chosen that were circular in cross-section; when oblique sections were measured, the diameters of the shorter and longer axes were averaged.

The number of seminiferous tubules was determined in 120 separate microscopical fields from 10 slides for each right and left testis of each of the 3 individuals in each age-group. The number of cells per tubule was also counted in one of the seminiferous tubules in each of these microscopic fields. To standardize the counts, tubules of circular section were chosen. Sections and microscopical fields in which shrinkage due to fixation was evident were avoided. Photographs of one of the sections in each age-group were taken with the projector objectives ×10 and ×30.

FIG. 1 (plate). Sections of testes. A, at 4 weeks, autopsied 2 Dec.
B, at 8 weeks, autopsied 28 Dec.
C, D, at 12 weeks, autopsied 25 Jan.

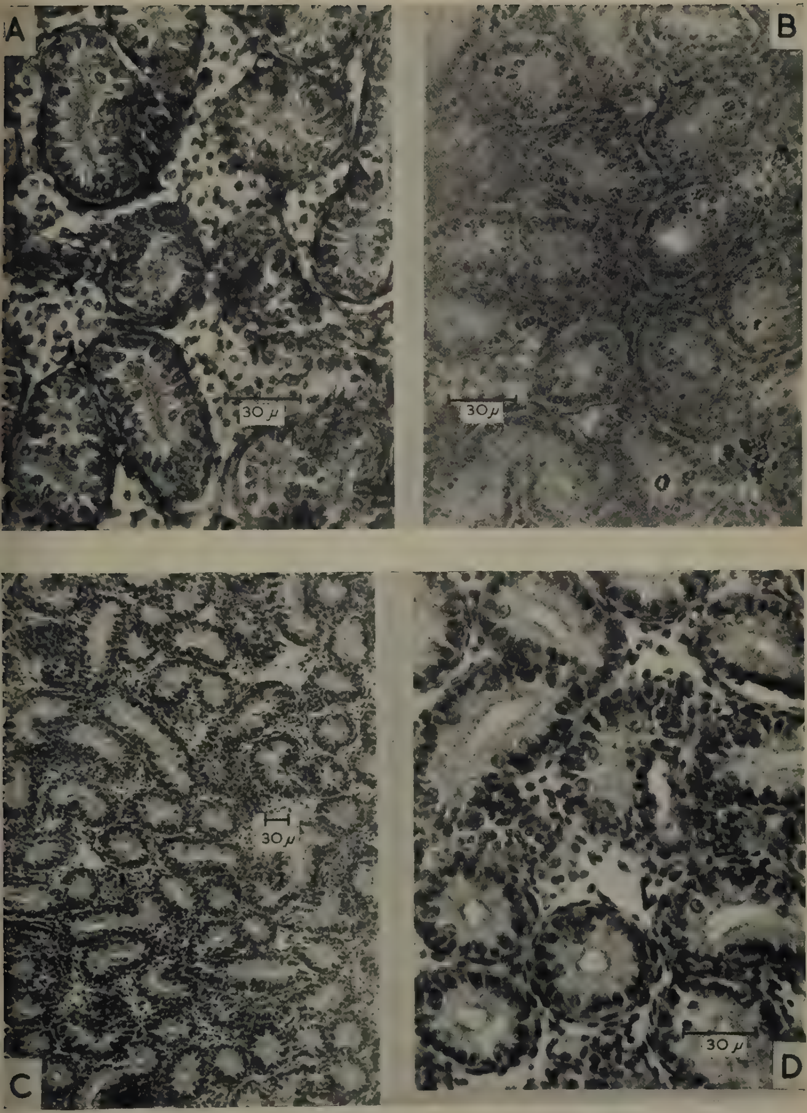

FIG. 1

G. A. R. KAMAR

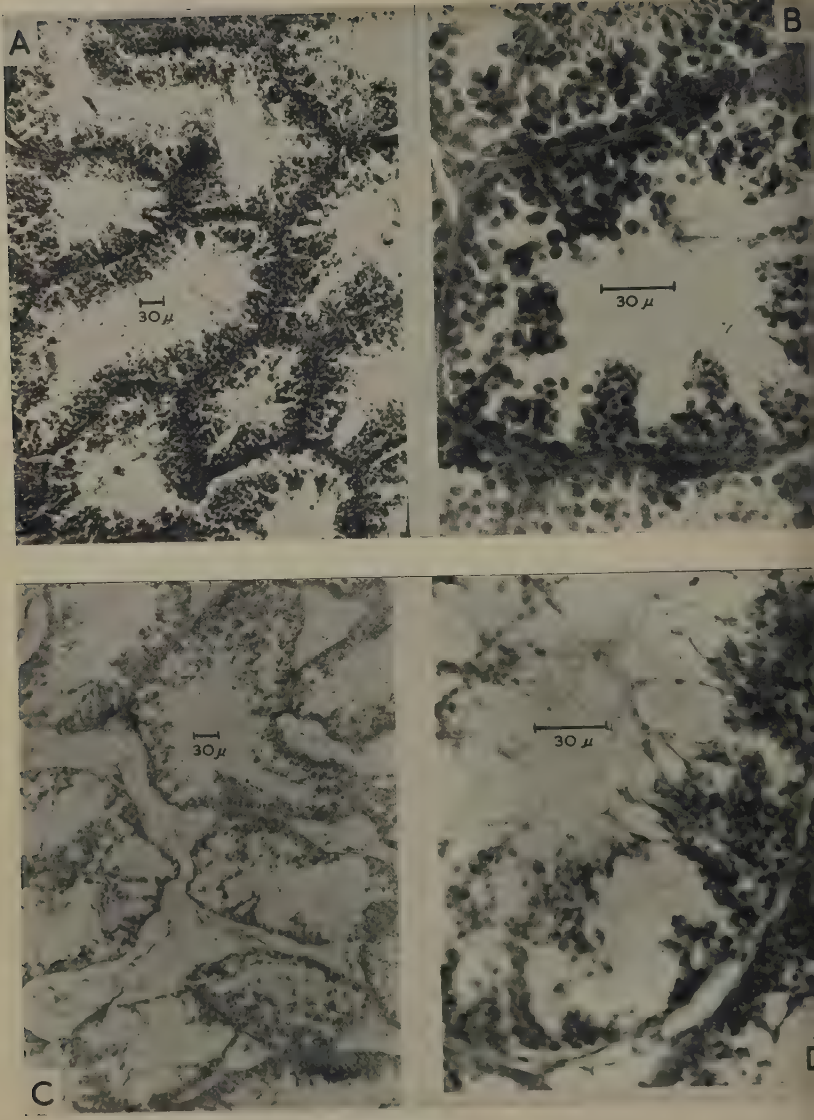

FIG. 2

G. A. R. KAMAR

RESULTS

At 4 weeks. Seminiferous tubules were lined for the most part with a single layer of spermatogonial cells. Primary spermatocytes were occasionally seen. In some tubules the lumen was beginning to form, and some were markedly coiled. The stroma was abundant and fibrous, full of interstitial cells (fig. 1, A).

At 8 weeks. Mitotic activity was now evident and the number of primary spermatocytes had increased. Approximately half the tubules showed a small lumen. The stroma, full of interstitial (Leydig) cells, was more abundant than at the previous age. The tubules were coiled, but the majority were still circular in cross-section (fig. 1, B).

At 12 weeks. Spermatogonial cells were more numerous and larger than before. Considerable numbers of advanced primary spermatocytes were also observed. In most tubules, the lumen was well developed. Interstitial cells were still abundant, and the stroma was extensive and fibrous (fig. 1, C, D).

At 16 weeks. The seminiferous tubules were now lined with several layers of cells: two layers of large spermatogonial cells, several layers of primary spermatocytes, and moderate numbers of secondary spermatocytes. A few spermatids were found in clusters with occasional spermatozoa among them. Sertoli cells were rare. The lumen was large and well developed in all tubules. Stroma and Leydig cells were now sparse but well formed (fig. 2, A, B).

At 20 weeks. In most regions the seminiferous tubules were now lined with 2 or 3 layers of spermatogonial cells. There were several scattered layers of primary and secondary spermatocytes, of which the secondary were the more abundant. Large numbers of spermatids were present, scattered in clusters. These were accompanied by moderate numbers of mature spermatozoa, extending towards the lumen and clumping round the scattered Sertoli cells. The lumen contained some loose spermatozoa. Most of the tubules were coiled and their cross-sections were elongated in shape. The large intertubular spaces contained only a few, well-formed, interstitial cells and but little stroma (fig. 2, C, D).

From 24 to 52 weeks. The variation in testis components in the different age-groups from 24 to 52 weeks was insignificant. The slight changes observed may indeed be due to other factors than age, such as seasonal variation in climatic or other environmental conditions. These age-groups, represented by sections at 28 weeks (fig. 3, A, B) and at 36 weeks (fig. 3, C, D), showed the highest spermatogenetic activity of all the age-groups studied. All stages of spermatogenesis were shown, with abundance of cells of all types, including ripe spermatozoa, and lumina full of spermatids and spermatozoa; the large intertubular spaces were full of well-developed interstitial cells and stroma.

It must be emphasized, however, that overlap occurs between the different phases in sections from different individuals at the same age. Cells of later

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FIG. 2 (plate). Sections of testes. A, B, at 16 weeks, autopsied 22 Feb. C, D, at 20 weeks, autopsied 29 March.

stages may be observed at earlier ages in the testes of certain individuals, and some sections depart from the general average of all the individuals.

Numbers of tubules and cells. The average diameters of seminiferous tubules at 4, 8, and 12 weeks, were 44, 45 and 59 μ, respectively (see Appendix, p. 405). The numbers of tubules per square cm at the same ages were 30,125, 31,940, and 20,067, with little variation. At these ages there was no indication of cell-division; the number of cells per tubule section was approximately constant. Over the period from 16 to 24 weeks of age, however, the tubules increased greatly in diameter, reaching values of 139, 150, and 215 μ at 16, 20, and 24 weeks respectively. At the same time, marked spermatogenetic activity occurred, resulting in increased numbers of cells, averaging 220, 232, and 536 cells per tubule section at these ages. Owing to the increase in tubule diameter, the number of tubules per square cm decreased markedly during this period, reaching values of 2,416, 2,292, and 1,513 respectively for the three ages of 16, 20, and 24 weeks.

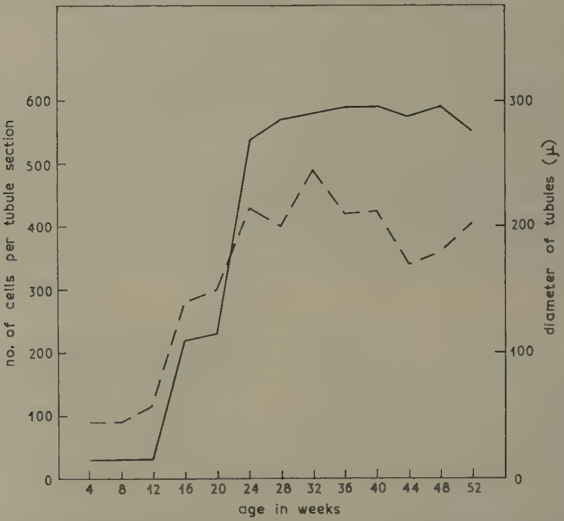

FIG. 4. Graph showing changes in the seminiferous tubules during development. Continuous line, number of cells per tubule section; broken line, diameter of tubules (μ).

When tubule-diameter or cell-number is plotted as ordinate and age as abscissa (fig. 4), a typical S-shaped growth curve is obtained for both. Three phases of tubule growth can then be distinguished: in the first phase tubule diameter and cell count are apparently constant, from the fourth to the twelfth week; the second phase, from 16 to 24 weeks, coincides with the

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FIG. 3 (plate). Sections of testes. A, B, at 28 weeks, autopsied 1 June.
C, D, at 36 weeks, autopsied 18 Aug.

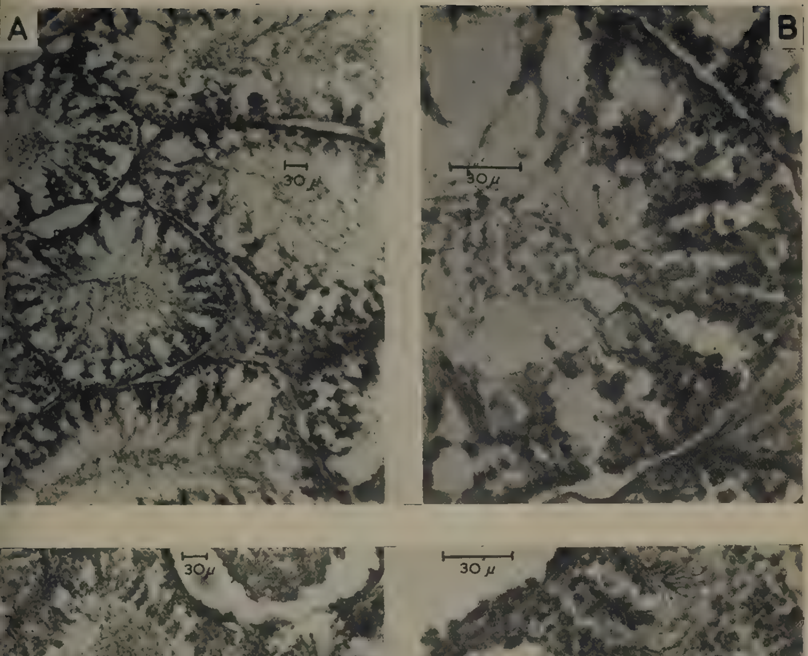

FIG. 3

G. A. R. KAMAR

period of greatest increase in testis weight; in the third phase, from 28 to 52 weeks, maturation processes are accompanied by increase in testis weight, but there is no systematic change in tubule size and cell number.

DISCUSSION

The period of greatest testis growth was shown to coincide with that in which the seminiferous tubules are at their largest and contain the largest number of cells per tubule section. The rapid increase in testis weight is mainly due, however, to increase in length of the tubules, rather than to increase in diameter. In other studies (Hiatt and Fisher; Kumaran and Turner; Charny, Conston, and Meranze), the formation of different types of spermatogenetic cells has been observed to occur in sequence throughout four stages. These stages were distinguished, however, by the incidence of successive stages in spermatogenesis, whereas the present study has distinguished developmental phases based on cell numbers and tubule-development. It is probable that four stages of spermatogenetic activity occur in Fayomi males as in other fowl. Phase I as defined here includes two stages of spermatogenesis, for example, while in phase II all stages of spermatogenesis are present.

APPENDIX

Histological changes in testes with age
(average of 3 males at each age)

Age in weeks	*Av. weight of pair of testes**	*No. of tubules per cm^2 of T.S.†*	*Av. diam. of tubule‡*	*Av. no. of cells per tubule section§*
	(g)		(μ)	
4	0·029	30,125	44	28
8	0·103	31,940	45	28
12	0·179	20,067	59	31
16	2·900	2,416	139	220
20	7·864	2,292	150	232
24	11·017	1,513	215	536
28	17·183	2,022	200	569
32	19·867	1,385	245	581
36	17·433	1,774	209	591
40	16·800	1,655	214	587
44	15·500	2,084	167	471
48	18·467	2,053	178	489
52	15·000	1,770	202	554

* Average weight of the pair of testes from one male obtained by dividing the total weight of right and left testes from three birds by three.

† Average number in 120 microscopic fields.

‡ Average of 100 seminiferous tubules.

§ Average of 120 seminiferous tubules.

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The Spermatophores of *Dolops ranarum* (Crustacea, Branchiura): their Structure, Formation, and Transfer

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SUMMARY

Sperm transfer in the genus *Dolops* involves the employment of spermatophores. In this respect the genus is unique within the Branchiura.

Proteinaceous wall material is secreted by arborescent spermatophore glands located in the carapace lobes of the male and is passed into a pair of long canals which terminate posteriorly as a pair of spermatophoric vesicles, part of whose walls are glandular. Spermatozoa from the vasa deferentia are injected into these vesicles and become surrounded by wall material. During the mating process the contents of each vesicle are squeezed out through the common genital orifice, where they become confluent and form a single, globular spermatophore. While its walls are still soft this is pressed against the base of the abdomen of the female, where it is pierced by two perforated spines at the end of the spermathecal ducts. The spermatophore hardens and remains attached to the female, thus giving the spermatozoa opportunity to migrate through the spermathecal ducts to the spermathecae. Not until after a moult of the female, however, during which the spermatophore is shed with the old cuticle, are the spermatozoa free to enter the eggs, each of which is believed to be pricked by the spermathecal spines during oviposition.

By virtue of the fact that it involves quite different organs and processes in the two groups, spermatophore formation indicates that the Branchiura and Copepoda are not closely related. On the other hand, the process is in no way suggestive of a close relationship of the Branchiura and Branchiopoda, and the isolated position of the Branchiura is emphasized.

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INTRODUCTION

THE employment of spermatophores during sperm transfer in the branchiuran genus *Dolops* has recently been demonstrated (Fryer, 1958). That spermatophores are formed by members of this genus is of particular interest in that hitherto the absence of these structures has been regarded as one of the diagnostic features of the Branchiura, and it is indeed well

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established that no such structures occur either in the well-known genus *Argulus* or in the less-known and entirely African genus *Chonopeltis*.

It is the purpose of the present paper to describe the structure and manner of formation of the spermatophores of *Dolops ranarum* (Stuhlmann), in which species they were first observed, and to give some account of the process of sperm transfer and associated aspects of the reproductive biology of this species.

MATERIAL AND METHODS

Specimens of *D. ranarum*, which has a wide distribution in the inland waters of Africa, and which occurs ectoparasitically on several species of fishes, were collected for the present study first in Lake Bangweulu Northern Rhodesia), which produced the material on which the original observations on spermatophores were made, and later in Lake Victoria, where material for detailed study was obtained. If kept in dishes of clean water, specimens removed from the host survive for several days and not infrequently mate under such conditions. Observations on spermatophore extrusion and transfer were made on such specimens. Dissections were made of both fresh and preserved material, and sections were cut from specimens embedded in low-viscosity nitrocellulose. Ordinary neutral 4% formaldehyde was found to be a better general fixative and to cause less shrinkage than Bouin's fluid, Heidenhain's 'Susa' fixative, or alcohol.

THE STRUCTURE OF THE SPERMATOPHORES

The general appearance of a spermatophore is best understood by reference to figs. 1 and 2. A spermatophore in position on the female is shown in fig. 1, A. When thus attached it strikingly resembles a minute pearl in form, colour, and lustre. A fully formed spermatophore is a subspherical or ovoid structure. At one pole it is perforated by two small, close-set, circular apertures, which occasionally have somewhat raised, crater-like rims, symmetrically arranged on each side of the meridional axis, and here too it gives rise to a projecting flange, whose exact shape varies slightly but which is basically a simple plate. The meridional axis of the spermatophore is not infrequently marked by a very shallow and indistinct furrow on its surface. The length of the longest axis of the spermatophore body varies from as little as 360 μ to as much as 650 μ. The smaller specimens tend to approximate more closely to a sphere than do the larger ones. The apertures have diameters of about 50 to 60 μ.

In relation to the size of the spermatophore its walls are extremely thick (up to about 45 μ) and in sections appear perfectly homogeneous in structure. In newly formed specimens the central cavity is completely filled with spermatozoa.

THE SPERMATOPHORE-PRODUCING ORGANS AND ASSOCIATED STRUCTURES

Before the process of spermatophore production can be described some account of the male reproductive system and of certain accessory organs of the

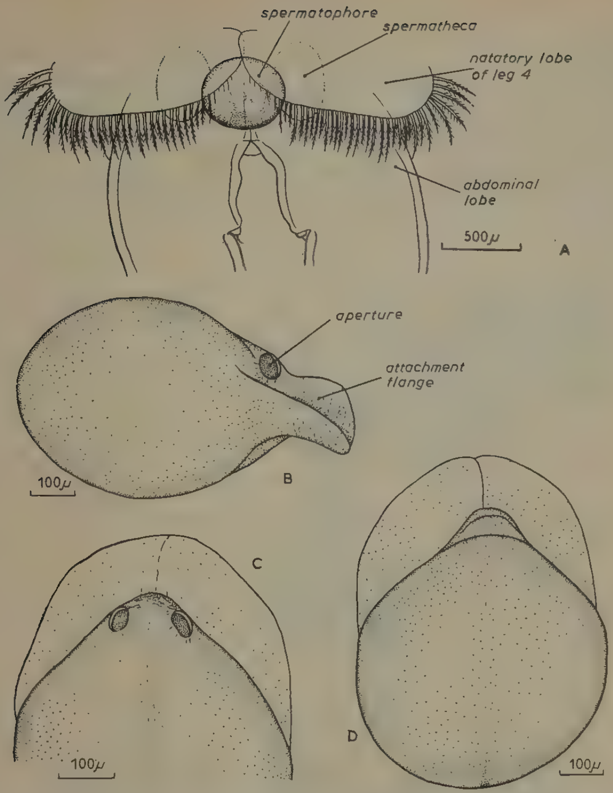


FIG. 1. A, spermatophore in position on female. B, detached spermatophore (lateral view). C, anterior end of a detached spermatophore from dorsal aspect, showing apertures. D, detached spermatophore (ventral view), as seen in position on a moulted cuticle.

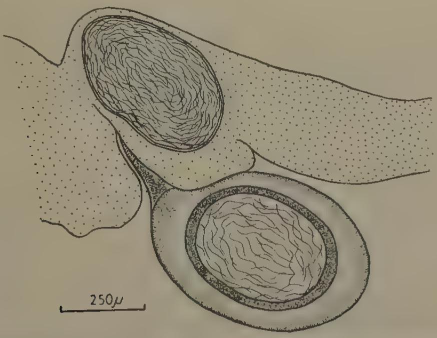


FIG. 2. Spermatophore in position. From a specimen embedded in L.V.N. and sliced to show the interior of the spermatophore and one of the spermathecae.

female system is necessary. Apart from casual statements, mostly by nineteenth-century investigators, usually referring to such details of the reproductive organs as could be discerned through the integument, the only account of the male system of any species of *Dolops* appears to be that of Maidl (1912) for *D. longicauda* (Heller). This is quite lucid, but his material was limited and as he was unaware that certain structures were concerned with the production of spermatophores he was unable to give a functional interpretation of his findings.

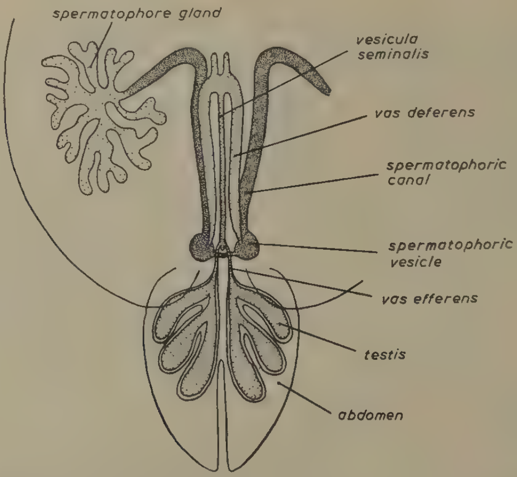
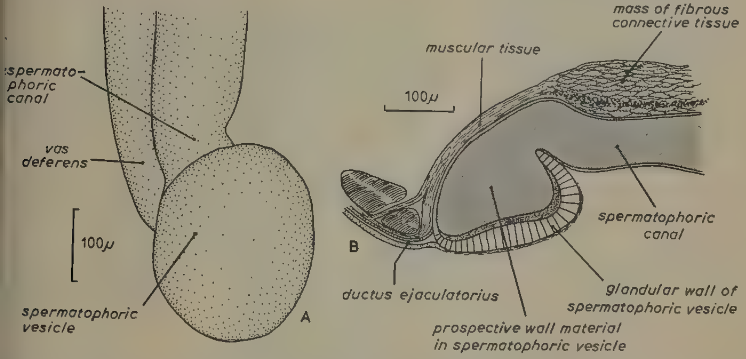


FIG. 3. Male reproductive system (semi-diagrammatic). The left spermatophoric canal is displaced and the spermatophore gland of that side is omitted.

The male reproductive system and the spermatophore-producing organs

The gross morphology of the reproductive organs is depicted in fig. 3. As in all Branchiura, the paired testes are located in the abdomen. In *D. ranarum* each testis has 3 lobes. From each there passes anteriorly a fine vas efferens, which quickly unites with its partner to form a much wider median duct which extends over the greater part of the length of the thorax and functions as a sperm reservoir or vesicula seminalis. At its anterior extremity this bifurcates and each branch doubles back on itself and, now somewhat greater in diameter than the vesicula seminalis, passes posteriorly as a vas deferens. Although morphologically vasa deferentia, these tubes in fact store far more sperms than does the vesicula seminalis. Extra space for sperm storage is provided by two short, anteriorly directed horns, located where the vesicula seminalis and vasa deferentia unite. In the region of the fourth pair of legs each vas deferens narrows and, in a manner described below, unites with a vesicular swelling which forms part of the spermatophore-producing apparatus. These swellings lie ventrally and can often be seen through the integument f the animal, particularly in living specimens (fig. 4, A). It was after observing them in this manner in several species of *Dolops* that Thiele (1904) amed them from the tubes with which they are continuous anteriorly as rostate ampullae', a term adopted also by Maidl (1912). Because of their nction they are best referred to as spermatophoric vesicles. From the dorsal rface of each arises a canal which runs forwards below and somewhat to the



IG. 4. A, a spermatophoric vesicle as seen in ventral aspect in the living animal. (The ductus jaculatorius cannot be seen.) B, semi-diagrammatic longitudinal section through a spermatophoric vesicle and the posterior end of a spermatophoric canal.

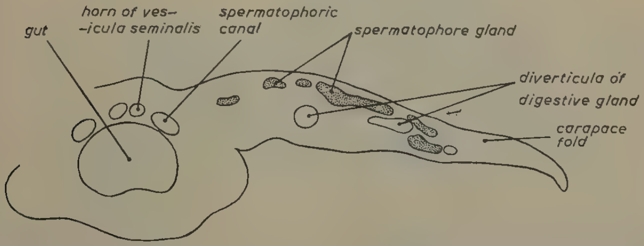


FIG. 5. Topographic sketch of a transverse section of a male, showing the distribution of the obes of the spermatophore gland in the carapace fold. The section is cut at the level of the horns of the vesicula seminalis.

outside of the vas deferens of its side as a wide tube, here designated the spermatophoric canal (fig. 4, A, B). This extends to about the anterior limit of the vas deferens before turning outwards at a rather acute angle and rapidly narrowing. Superficial examination, even of living specimens, usually gives the impression that this is a blind tube, but in fact it terminates in an arborescent glandular mass which ramifies through the carapace lobes and for the most part overlies the digestive diverticula of the alimentary canal (fig. 5). This glandular structure produces the spermatophore wall material and therefore merits the name spermatophore gland. Each spermatophoric vesicle is also continuous with a short, inwardly directed, narrow, chitin-lined ductus ejaculatorius; both of these open at the single median genital aperture. This duct is usually closed and cannot be made out except in sections. The vesicula seminalis and vasa deferentia are usually packed with sperms, whose axes in general lie parallel to those of the ducts they occupy. The lumen of each spermatophoric canal is filled with a structureless, white secretion, which stains in a uniform manner with a variety of stains. This is the material of which the spermatophore wall is composed. In living specimens peristaltic movements can sometimes be observed in the vesicula seminalis and in the vasa deferentia, but have not been observed in the spermatophoric canals.

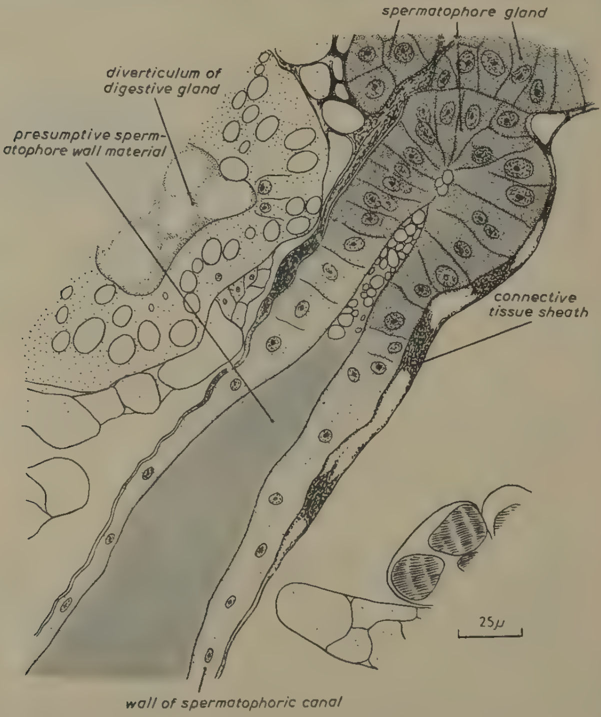

spermatophore gland
diverticulum of digestive gland
presumptive spermatophore wall material
connective tissue sheath
25μ
wall of spermatophoric canal

FIG. 6. Sagittal section through the region where the spermatophore gland unites with the spermatophoric canal. The white areas represent blood-filled haemocoelic spaces. (The droplets present at the union of gland and canal are believed to be artifacts, but it is just possible that they represent a fluid stage in the secretion of wall material.)

Histologically the spermatophore gland consists of a system of branched ducts surrounded by glandular cells, whose cytoplasm and large spherical nuclei both stain deeply with haemotoxylin (figs. 6, 7). There are no intrallular ducts. The intercellular ducts unite and finally discharge into the wide ermatophoric canal, with the cells of whose walls the gland cells are in irect continuity. The transition from gland lls to canal wall-cells is gradual as can be seen om fig. 6. The cells gradually become flatter nd less deeply staining, their nuclei become maller and more compressed, and the cell oundaries become less and less distinct, so at the canal wall appears to be syncytial. hroughout most of its length the wall is so ompressed that it is difficult even to detect the resence of nuclei. It is apparent, therefore, hat the canal serves purely as a storage chamber r the secretion produced by the spermato-hore gland. The same cannot be said of the permatophoric vesicle into which this secretion asses, for its wall, particularly in its ventral and outer lateral regions, is hick and consists of darkly staining columnar cells, several of whose nuclei ontain two nucleoli (fig. 10, A, B). These are very obviously glandular in unction, as their structure suggests, for in the region of their occurrence, nd between them and the secretion which fills the spermatophoric vesicle, ccurs another secretion with somewhat different refractive and staining roperties (fig. 10, B). This is discussed below.

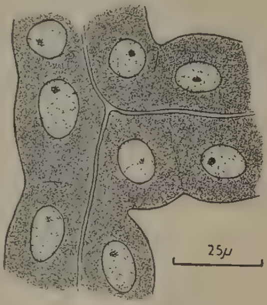
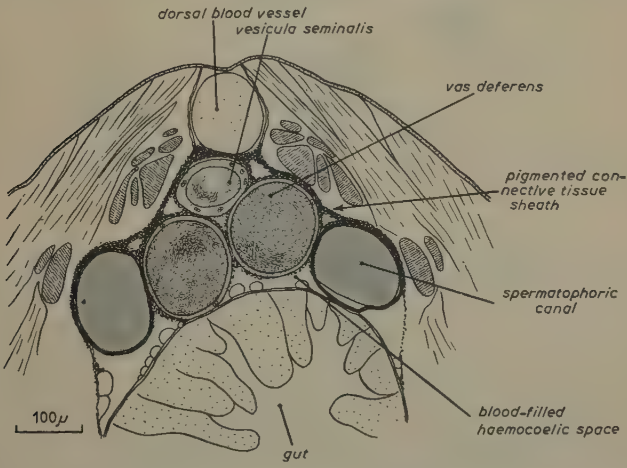


FIG. 7. Details of part of the spermatophore gland.



IG. 8. Transverse section of the male reproductive system a short distance anterior to the spermatophoric vesicles.

The histological nature of the walls of the sperm-carrying canals differs from region to region. The wall of the vesicula seminalis is thick and nuclei are readily apparent, but cell-walls are not often distinct. Over much of their length the vasa deferentia have similar though rather thinner walls. Towards their posterior extremities, however, considerable changes take place over a very short distance. It is here that each vas deferens unites with the spermatophoric vesicle of its side and in this region each is modified to form a mechanism for the injection of sperms into the mass of prospective spermatophore wall material contained within the spermatophoric vesicle. As the vas deferens approaches this region it rapidly narrows and its walls thicken somewhat (compare text-figs. 8 and 9, A). The narrowing of the lumen continues, the wall-cells, and particularly those located ventrally, enlarge and tend to become vacuolate, and their boundaries become distinct (fig. 9, B). At the level of the anterior extremity of the spermatophoric vesicle the lumen of the vas deferens is very narrow and the duct is somewhat compressed laterally, so that the enlarged ventral cells of its walls tend to reach downwards towards the region where the spermatophoric canal gives rise to the vesicle (fig. 10, A). This process continues, the lumen of the vas deferens becomes slit-like, and the ventral portion of its wall, now composed of vacuolate columnar cells, ultimately

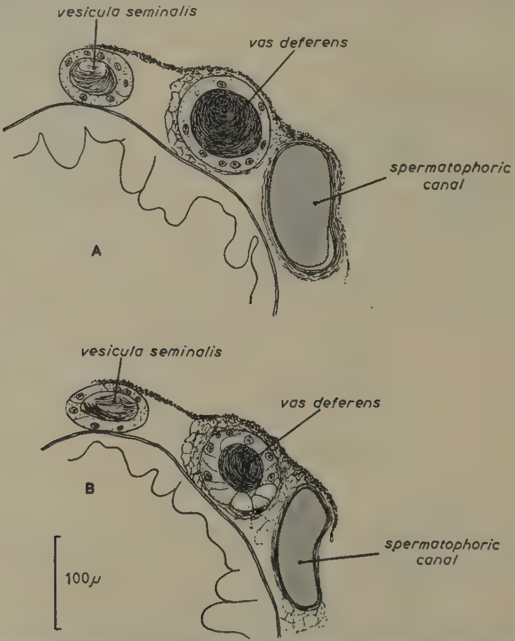


FIG. 9. A, transverse section of the left side of the male reproductive system immediately anterior to the spermatophoric vesicle. B, the same, a little further posteriorly.

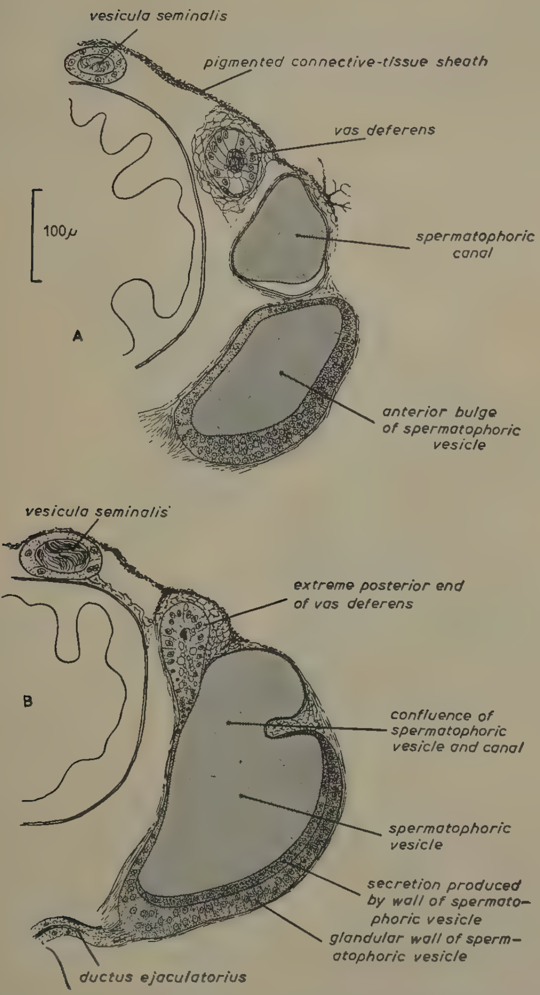

vesicula seminalis
pigmented connective-tissue sheath
vas deferens
100μ
spermatophoric canal
A
anterior bulge of spermatophoric vesicle
vesicula seminalis
extreme posterior end of vas deferens
B
confluence of spermatophoric vesicle and canal
spermatophoric vesicle
secretion produced by wall of spermatophoric vesicle
glandular wall of spermatophoric vesicle
ductus ejaculatorius

FIG. 10. A, transverse section of the left side of the male reproductive system, passing through the extreme anterior portion of the spermatophoric vesicle. B, the same, a little further posteriorly, showing the region of confluence of the vas deferens and the spermatophoric vesicle.

unites with the wall of the spermatophoric vesicle (figs. 10 B, 11). The lumen of the vas deferens thus opens into that of the spermatophoric vesicle.

All the organs of the reproductive system including the spermatophore glands are encased in a fibrous connective-tissue sheath, apparently continuous with the basement membrane. This fulfils several functions. First it serves as a means of suspending the various organs from the tergites of the thorax. It also forms a canopy over these organs in which is deposited much dark pigment (figs. 4 B, 8–10). Such pigment develops, often in very definite patterns, above the uteri of many female Branchiura and over the testes of males—a distribution which suggests that it serves to protect the genital products from the effects of light. The rigidity of this fibrous tissue also enables it to function both as an 'endoskeleton' which gives support to delicate structures and as a tissue from which slips of muscle can take their origin. Both the latter functions are exhibited by the connective tissue in the region where the vas deferens forms the injector of sperms (fig. 11). Here the sheath supports the delicate vas deferens and to the outside is continuous with a strip of muscular tissue whose contractions, by pulling down the overlying sheath, will deform the posterior extremity of the vas deferens and, assisted by the peristaltic movements of the more anterior regions of the duct, will result in the discharge of sperms, which are in effect injected into the spermatophoric vesicle. In only one of the sectioned specimens was injection actually in progress, but in this

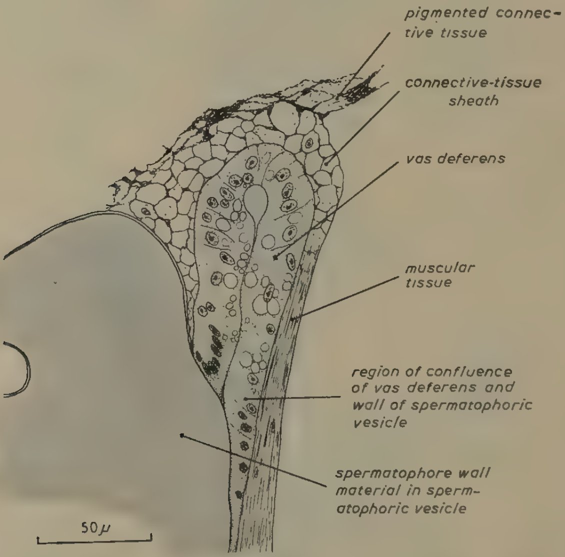

pigmented connective tissue
connective-tissue sheath
vas deferens
muscular tissue
region of confluence of vas deferens and wall of spermatophoric vesicle
spermatophore wall material in spermatophoric vesicle
50µ

FIG. 11. Details of the injection apparatus at the region of confluence of the vas deferens and the spermatophoric vesicle.

pecimen, although histological details were poorly defined, the injection was ery clearly shown (fig. 12). The injected sperms are arranged in a compact ass or cord. This will facilitate their penetration into the wall material and, y ensuring that they cohere for a time, will enable the wall material to flow round them. It may be that the vacuolated cells at the extreme posterior nd of the vas deferens are secretory. If this is not so, then it is difficult to nderstand whence the fluid which is undoubtedly present in newly formed permatophores is derived.

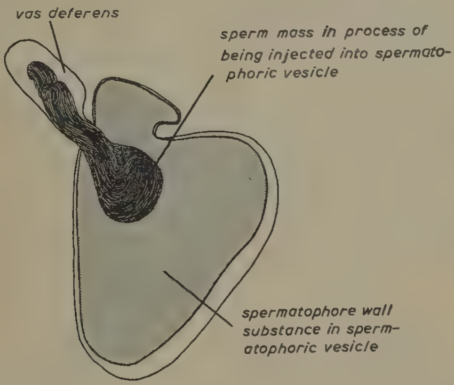

vas deferens
sperm mass in process of being injected into spermatophoric vesicle
spermatophore wall substance in spermatophoric vesicle

FIG. 12. The injection of spermatozoa into the spermatophoric vesicle.

A rather similar mechanism is concerned with the discharge of the spermatophoric vesicles. The dorsal wall of each of these is overlain by muscular tissue which takes its origin in a local accumulation of fibrous connective tissue, itself slung from the tergites of the thorax which overlie the posterior end of the spermatophoric canal. This muscle is inserted on the ductus ejaculatorius and its contraction will result in compression of the spermatophoric vesicle, the opening of the ductus ejaculatorius, and the consequent extrusion of the spermatophore mass.

The female reproductive system in relation to spermatophore transfer

Certain organs associated with the female reproductive system fulfil an important function during spermatophore transfer and require description before the mating process is described. Besides possessing a single functional ovary whose contents are discharged through a single median aperture, the female reproductive system includes two quite separate spermathecae. These take the form of ovoid sacs located at the base of the abdomen. They have no direct connexion with the female reproductive system proper and, as in *Argulus*, each communicates with the exterior by way of an extremely fine and somewhat coiled duct leading inwards towards the bottom of the cleft between the abdominal lobes, where each terminates in a minute but sharp

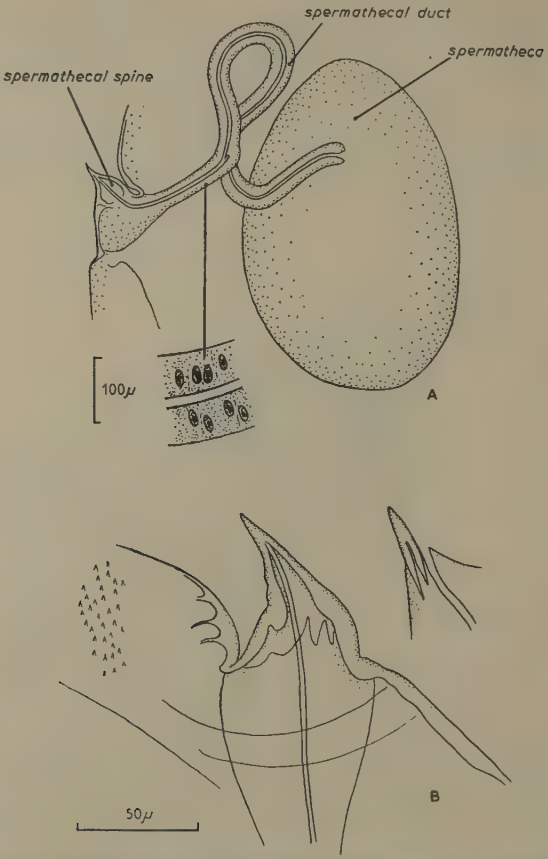


FIG. 13. A, one of the spermathecae of the female and its associated duct and spine. The inset shows cellular details of the spermathecal duct. B, a spermathecal spine. The inset shows a different (and somewhat enlarged) view of the extreme tip and the aperture of the duct.

and sclerotized, perforated spine (fig. 13, A). These ducts and spines are here referred to as spermathecal ducts and spines respectively. The ducts have an extremely narrow lumen lined with cuticle and this is surrounded by a thick and apparently glandular wall. About a third of the way between the spermathecal spine and the spermatheca is the suggestion of a blind diverticulum similar to but very much shorter than that to be seen in certain species of *Argulus*, and here the wall of the duct is somewhat thickened. Coiling of the duct, which permits considerable freedom of movement, is doubtless necessitated by the mobility of the abdomen, which here contains many loose parenchymatous cells, and by the need to allow movement of the spermathecal spines. Around the base of each spermathecal spine is a region of thickened cuticle, between which and the spine itself a region of flexible cuticle permits the spine to be pointed in various directions. Near the base of the spine and somewhat posterior to it the cuticle of the abdomen is roughened by the presence of a number of minute spinules (fig. 13, B), whose significance becomes apparent when spermatophore attachment is studied.

The spermathecae have a cuticular lining. Obviously, however, there is no means whereby the animal can completely rid itself of this cuticle at times of ecdysis. At each moult, therefore, the old lining which, judging from the fact that it is very thin in comparison with the functional cuticle, must undergo a considerable amount of digestion during the pre-moulting period, apparently splits along its longitudinal axis and is shed into the cavity of the spermatheca. By some means, not ascertained, successively moulted cuticles adhere to each other to form a compound structure which, when a spermatheca is dissected, readily slips out from within the functional cuticle. When flattened this structure exhibits distinct annuli, which presumably enable one to determine the age of the parasite, at least to the extent of ascertaining the number of ecdyses through which it has passed since the spermathecae were formed. Up to 8 annuli have been counted in large specimens.

SPERMATOPHORE EXTRUSION AND TRANSFER

Extrusion of a spermatophore does not take place until the mating process has begun. While absolute proof is lacking, the inference is that in nature mating takes place while the parasites are on the host. That mating sometimes takes place away from the host cannot, however, be ruled out, for all the present observations were made on specimens which had been removed from the host and which on several occasions produced and successfully transferred spermatophores under these conditions.

During the mating process the female is seized by the male, which, in the cases observed, was always smaller than the female. This is probably the most usual state of affairs in nature, for mature females are usually larger than males. The female is gripped by the maxillulary hooks normally used for attachment to the host. These at first grip whatever portion of the female is available, but very quickly the male works itself into such a position that it overlies the female, its ventral surface being in contact with the dorsal surface of the female, and its hooks grip convenient points of the carapace. By slewing sideways either to the right or left the male manœuvres itself into such a position that the abdomen of the female can be gripped between legs 2 and 3. On the anterior face of the basal segment of leg 3 is a roughened projection, which doubtless facilitates this part of the proceedings. (On one occasion the abdomen was held for a time between legs 3 and 4.) A female ready for mating assists in this by lifting the abdomen. The male then flexes its body so as to bring the ventral surface of the abdomen, and hence the genital orifice, face to face with the comparable structures of the female. Not until this state of affairs has been attained does extrusion of the spermatophore begin, though the spermatophoric vesicles are very conspicuous at the beginning of mating as though they are bulging with material ready for discharge. The bulging may be an indication that sperms have been injected into the spermatophoric vesicles as a result of the stimulus given by the preliminaries of the mating process. At all events, as a result of the injection process described above, at the beginning of mating such a spermatophoric vesicle contains a mass of sperm surrounded by prospective wall material. Nipping off of this mass from the rest of the wall material in the spermatophoric canal is probably assisted by contractions of the muscular sheath which is present at the extreme posterior end of the spermatophoric canal but not elsewhere (compare fig. 9, A, B with fig. 8). Extrusion is initiated simultaneously in each spermatophoric vesicle. From each a quantity of prospective wall material (fig. 14, B) is exuded along its ejaculatory duct. There is presumably a core of spermatozoa within this wall material. Extrusion is probably assisted by contraction of the dorsal muscular tissue described on p. 417, possibly with the assistance of a

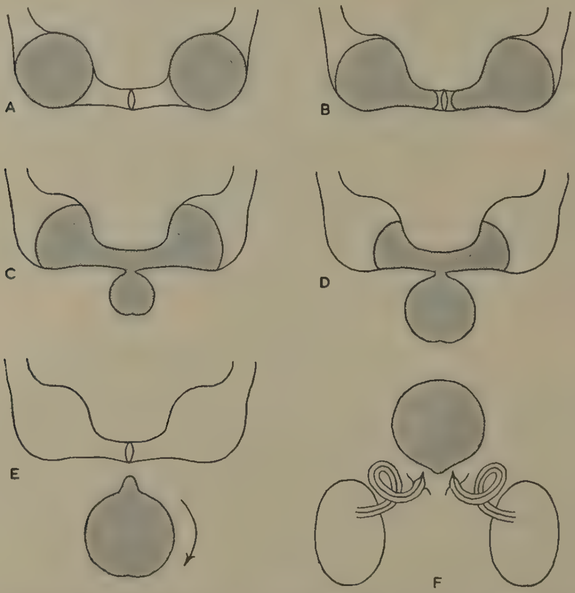


FIG. 14. Diagrammatic representations of spermatophore extrusion. A, the situation at the outset. B, extrusion begins and the material flows along the ductus ejaculatorius. C, material passes to the exterior through the single genital orifice as a single globule. D, the spermatophore increases in size. E, extrusion is complete. The arrow indicates how the complete spermatophore is rotated during transference to the female. F, the spermatophore is pushed against the spermathecal spines of the female.

local increase in the pressure of the haemocoelic spaces. The two exudates reach the genital orifice at the same time and there become confluent and pass to the exterior as a small globule (fig. 14, C). This then increases in size (fig. 14, D). At first its dual origin is clearly marked by a meridional furrow, but as the size increases still more and the wall material of each side mingles this becomes less and less distinct (fig. 14, C–E). When the whole of the material from the spermatophoric vesicles has been exuded, a process which takes only 4 or 5 sec, the now more or less globular and completely sealed spermatophore is kicked away from the genital orifice by one of the fourth pair of legs. The last part of the spermatophore to leave the male genital orifice is drawn out into a small papilla which can be seen to be still very plastic (fig. 14, E). The spermatophore is then kicked round by the fourth pair of legs and the *last* part to emerge is pushed against the female (fig. 14, F). Although this cannot be observed at the time, subsequent examination reveals that during this part of the process the soft portion of the spermatophore is pierced by the spermathecal spines of the female and that these remain embedded in the wall (fig. 15, A, B). It is by this piercing of the wall while it is still soft that the two apertures of the fully formed spermatophore are formed. It appears that the fourth pair of legs of the male hold the spermatophore in position against the female for some time after first attachment. It is presumably during this period that the still very plastic wall material in contact with the female cuticle moulds itself to the shape of that cuticle and, by the forces of surface attraction, flows out to form the attachment flange. This explains why a spermato-

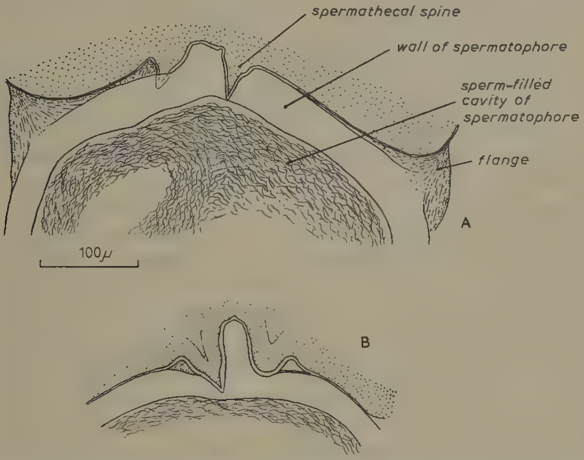


FIG. 15. A, T.S. through a spermatophore in position on a female, showing how the spermathecal spines penetrate its thick wall. (Note how the wall material in this region moulds itself to the shape of the female and fills the gap between the spermathecal spines.) B, the same, to demonstrate further the moulding of the wall at points of contact with the female.

phore always fits exactly the female to which it is attached although there is considerable disparity in size both among females receiving spermatophores and among the spermatophores themselves. The roughened nature of the cuticle near the base of the spermathecal spines described above doubtless serves to assist in the firm anchoring of the spermatophore.

The male then releases its hold on the female and the mating individuals separate, the female carrying away the now firmly attached spermatophore. The spermatophoric vesicles of the male no longer bulge with material but the spermatophoric canals are by no means emptied.

No 'former' or moulding organ is involved in the formation of a spermatophore and the form assumed by the latter is fairly readily explained by basic physical principles. Confluence of the wall material from each side and of the two sperm masses must take place in the same way as that in which two soap bubbles become confluent on contact. When this has taken place, the situation during subsequent extrusion is one in which an incompressible fluid (the sperm mass) is surrounded by a soft, deformable, and, to some extent at least, extensible sheath (the spermatophore wall) of homogeneous and isotropic material. Such a mass will, as a result of the mechanical forces (surface tension) operating in such a system, tend to give rise to a spherical structure just as soap bubbles and drops of fluids tend to be spherical. An abnormal event greatly clarified this and other aspects of spermatophore structure. On one occasion a mating male and female were disturbed when an attempt was made to move them under a microscope, and the female turned on its back. Mating nevertheless continued and exceptionally clear observations on spermatophore extrusion were made. The male, however, failed to attach the spermatophore and the structure fell away as a completely sealed and almost perfect sphere, whose even contours were marred only by the faintest trace of a papilla at that portion of the surface which last left the male genital orifice.

Under normal conditions other mechanical forces operate and lead to deformation of the spermatophore from the spherical form. Differential hardening of the wall is probably unimportant because of the rapidity of extrusion, but attachment at one point brings into play forces other than surface tension and explains why the free surfaces of a spermatophore are not part of a perfect sphere.

The way in which the flange is formed has not been observed, but it is apparent that this structure is derived from the last, and therefore the most fluid, portion of the spermatophore to emerge from the genital aperture. Contact between this and the cuticle of the female will bring into play attractive forces such as can be simulated for instance by dipping part of a small ball-bearing into Canada balsam and then placing it on a hard surface. Extension in one direction of the broad 'contact base' thus formed can be achieved if there is opportunity for the balsam to flow between two closely apposed surfaces—as it does between a slide and slightly raised coverslip. (By such a simple set-up a state of affairs remarkably similar to the spermatophore and its flange can be produced.) Such a possibility also presents itself to the soft part of a spermatophore, as can be seen from fig. 2, in which, incidentally, the gap occupied by the flange does not necessarily represent the width at the time of its formation. It is possible that the secretion produced by the walls of the spermatophoric vesicles is responsible for the formation of the flange of the spermatophore. This material, whilst still contained within the vesicles, maintains its own individuality and, as shown by this and its somewhat different staining reactions, must have at least slightly different properties from the rest of the wall material. The impression gained from sections is that this material is more fluid than the main secretion. On the other hand, apart from the fact that the flange sometimes becomes rather brown after preservation in formalin, which may merely reflect its thinness, it and the walls of the body of the spermatophore appear to be of the same composition. If the entire structure is composed of material produced in the spermatophore glands, then it can only be suggested that the secretion of the spermatophoric vesicles either serves as a lubricant during spermatophore extrusion or assists in some unknown way in the hardening or waterproofing of the walls. No outer layer can, however, be detected in sections of the walls.

No dissolution of any part of the spermatophore on the part of the female is necessitated. Whether any female secretion facilitates flow of the flange material is not known, though there is some darkly staining glandular tissue in the region of the spermathecal spines which could conceivably produce such a secretion. Apart from this possibility the part played by the female is purely that of mechanically puncturing the spermatophore by the spermathecal spines.

Although, because of the opacity of their thick walls, it is quite impossible to observe the sperms within the spermatophores, it was found that, by subjecting the completely sealed spermatophore mentioned above to intense reflected illumination, changing patterns of reflection could be observed, showing that the sperms were active within and suggesting continued movement in one direction. Ruptured spermatophores also contain active spermatozoa. It appears, therefore, that activity of the sperms commences as soon as the spermatophores are discharged, and that some seminal fluid is injected into the spermatophoric vesicles with the spermatozoa. Vigorous and continuous activity takes place within the spermathecae, through the walls of which circulation of the sperms can be readily observed.

It is obvious that sperms migrate from the spermatophores through the spermathecal ducts to the spermathecae, but the mechanism whereby this is accomplished is open to debate. The cavity of the spermatophore contains nothing which could swell and push out sperms in the way in which they are pushed out of the spermatophores of certain crickets (Khalifa, 1949*a*), nor are any of the sperms used for this purpose as they are in at least some calanoid copepods (Heberer, Lowe, cited by Marshall and Orr, 1955). Chemical attraction of the sperms to the spermathecae seems the most plausible explanation, but it involves one difficulty. As the sperms arrive they must displace some of the presumed attractive fluid which will flow into the spermatophore, thereby to some extent reducing the relative attraction of the spermathecal fluid. Even in spermatophores cast off at ecdysis, however, some sperms remain, so it may well be that a sufficient number to ensure efficiency is attracted to the spermathecae before the attractive gradient becomes too small to be effective. Other theories, which demand pumping movements on the part of the spermathecae, are less satisfactory; for unless the two operate antagonistically, for which there is no evidence, the setting up of a negative pressure in the spermatophore, even if only momentarily, is involved.

General Remarks on Reproduction

As the only exit from the spermathecae of the female is through the spermathecal ducts, it follows that the spermatophore must be shed before sperms become available for fertilization of the eggs. In other words, a female carrying a spermatophore can never deposit fertile eggs. Barring accidents, for which no evidence was obtained, the only way in which a female can rid itself of a spermatophore is by moulting. During this process the spermathecal spines and the linings of the spermathecal ducts are shed with the general cuticle, and the spermatophore, being firmly attached to the latter, is shed with them. A route to the exterior is now open to the sperms.

It is generally assumed that in argulids generally, the spermathecal spines are used for the penetration of the eggs as, one by one, they are deposited, and that by this means each egg receives an injection of sperms. There seems to be no reason to doubt this, for although the actual injection does not appear to have been observed, there are no other apparent means whereby the eggs, which have very thick shells and lack a micropyle, can be fertilized. In *D. ranarum* oviposition takes place in dishes with some reluctance, but has been observed at least in its gross aspects, though it has not been possible to make out the part played by the spermathecal spines during this process, as these are very small and inconveniently situated for observation. However, the observations made it clear that during oviposition each egg must pass beneath that portion of the abdomen on which the spermathecal spines are located and that it would be difficult for the latter to avoid touching the egg surface. As the study of spermatophores reveals, the spermathecal spines are capable of penetrating quite thick objects and it is easy to believe that some of the considerable muscular convulsions which accompany oviposition assist in driving them through the egg-shell. Very slight compression of the spermathecae would be sufficient to inject a few sperms into each egg.

The female reproductive cycle therefore consists of mating, ecdysis, and oviposition, in that order. Further moults can take place without interfering with oviposition, provided that sperms are still available, but after each mating the cycle must be repeated and follow the above sequence.

A Note on the Spermatophore Wall Material

Certain of the properties of the spermatophore wall material are evident from the above account, and are supplemented here by brief notes on other physical and chemical properties. Precise quantitative terms cannot be applied o the physical properties, but it is hoped that the purely descriptive terms sed here will convey a general impression of the nature of the wall material.

If a full spermatophoric canal is removed from a fresh specimen and com-ressed between a slide and cover slip, wall material flows slowly out and xhibits the fluidity to be seen at spermatophore extrusion. From fresh naterial of *D. ranarum* it is possible also to remove the secretion contained in he spermatophoric canals by use of a pair of very fine forceps. The entire ontents of a canal can be extracted through the posterior end of the canal by his means, and it is then possible to observe the material from which sper-natophores are constructed. Whether removed 'dry' or under water the naterial is usually sufficiently viscous to permit extraction in a single piece nd it emerges like an elongated white worm. It is very viscous, somewhat ticky, ductile, and fairly elastic. If a little of this material is stretched into a ong thin strand, say 50 to 70 μ in diameter, without coming into contact with vater, it appears almost colourless by both reflected and transmitted light. f water is added a surface reaction takes place and, within a minute or so, the naterial becomes grey by transmitted and silvery by reflected light. This hange in optical properties seems to be due to a very fine puckering of the urface layer of the material, which can be followed under the microscope. resumably the same reaction takes place during spermatophore formation nd is the first process in the hardening of the wall.

Although extracted wall material kept immersed in water remains quite oft for several hours, its property of ductility is almost completely lost in less han 30 min. Old spermatophores are still 'soft' in so far as the surface admits f permanent indentation if pressed with the point of a fine needle. If teased by ine needles, old spermatophores rupture in an irregular manner but are not trikingly brittle nor do they have the 'toughness' which one associates with ncalcified cuticular structures in arthropods. In specimens fixed in formalin which seems to toughen them) the walls are, however, sufficiently strong and igid to permit clean bisection with a sharp, fine scalpel.

Spermatophores are not chitinous but proteinaceous in composition. This s shown by their complete dissolution in a hot saturated aqueous solution of otassium hydroxide and by the strongly positive result which they give to he xanthoproteic test. They also give a positive result to the argentaffin test nd dissolve in a solution of sodium hypochlorite. Only a 1% commercial pre-aration of the latter was available (Brown (1952) recommends a 10% solu-ion), but even in this they dissolved within a few hours with the evolution of as bubbles. These tests, and the failure of the spermatophores to be dis-ersed in boiling water, dilute hydrochloric acid, dilute caustic alkalis, cold aturated aqueous solutions of caustic alkalis, or a solution of calcium chloride, ndicates that they are composed of quinone-tanned protein, though their olour differs from that of most such tanned proteins. They dissolve com-letely and within a few minutes in cold concentrated hydrochloric acid but re not dispersed completely by cold concentrated nitric and sulphuric acids. n nitric acid they become brown and translucent but do not dissolve even after several hours' immersion, while in sulphuric acid they rapidly collapse and fragment and become a rich red brown, but again persist in this state even after several hours' immersion. The application of heat, however, causes rapid solution in these acids. In the case of sulphuric acid the resulting solution is wine red in colour.

A test for lipids (Sudan black) gave a positive result even after the spermatophore had been immersed in xylene for a few hours. The presence of lipids, apparently firmly bonded to the protein, may assist in waterproofing the spermatophore. In spermatophores of the terrestrial cricket *Gryllus* (*Acheta*) *domesticus* L., Khalifa (1949*a*) found no trace of lipids.

Change of the wall material from the fluid to the solid state is reflected by a change in staining properties, for it was observed that while the secretion in the spermatophoric canals stained easily with haematoxylin, similarly treated sections of the spermatophore wall stained much less readily.

After it had been discovered that the spermatophore wall material gives the reactions of a quinone-tanned protein (p. 425), it was thought that the glandular tissue of the spermatophoric vesicle might secrete the aromatic material which tanned the protein secreted by the spermatophoric gland, much as one gland secretes protein and the other the tanning orthodihydroxyphenol during the formation of structures as diverse as the ootheca of cockroaches (Pryor, 1940) and the byssus of the lamellibranch *Mytilus* (Brown, 1952). However, this appears not to be the case, for material that had been carefully removed from the spermatophoric canal and had never been in contact with or even in proximity to the glandular wall of the spermatophoric vesicles, gave just as positive a result to the argentaffin test as did material from the vesicles themselves.

Quinone-tanned proteins tend to be dark, but the spermatophores of *D. ranarum* have a pearly lustre. This may indicate a fine lamination of the wall—a lamination in which layers of one refractive index are separated by layers of different refractive index, so that pearliness arises from multiple reflections from thin films, as in real pearl. (I am indebted to Dr. L. E. R. Picken for this suggestion.) The transmitted colour of the wall is thus obscured. When a piece of the wall is immersed in water and examined by transmitted light under the microscope, however, surface reflection is abolished and the wall is seen to be very pale amber.

THE SPERMATOZOA

Stuhlmann (1891) referred to the spermatozoa of *D. ranarum* as being *fadenformig*. This is an apt description of a fully mature spermatozoon. Complete maturity, however, seems usually, but by no means invariably, to be delayed at least until the sperms arrive in the spermathecae of the female, and even there incompletely mature sperms are often to be found.

A fully developed sperm has the general appearance of a minute nematode worm and has a length of about 138 to 165 μ. There is absolutely no head. The diameter of the sperm body (the term 'flagellum' seems inappropriate in his case) is about 0·7 μ, but it is difficult to measure this object accurately. In many specimens almost all the sperms present in spermatophores and many of those in the spermathecae of the female appear at first sight to have a dis-inct head. In some this appears to take the form of a simple flattened swelling

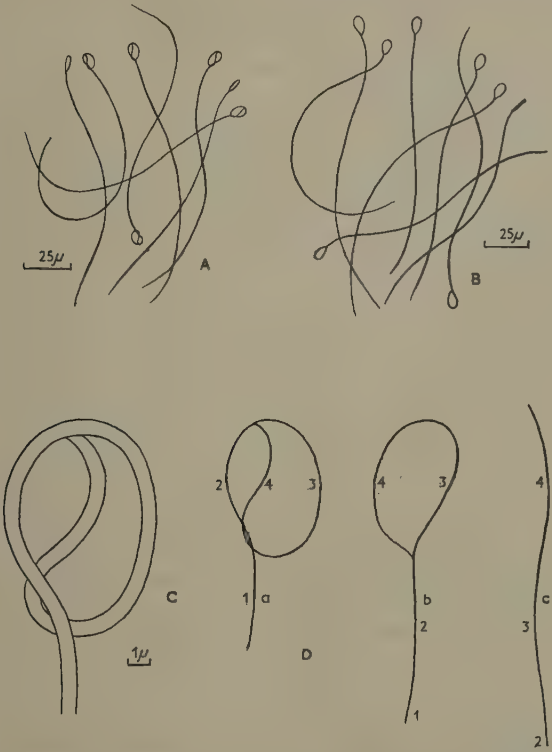


FIG. 16. A, spermatozoa at stage 1 of maturity. For explanation see text. B, spermatozoa at tage 2 of maturity. For explanation see text. C, the tip of a spermatozoon at stage 1 of maturity, howing coiling. (Owing to the difficulty of measuring the diameter of the sperm the scale is nly approximate.) D, diagrams illustrating the uncoiling of the tip of a spermatozoon. The same region is denoted by the same number in each case.

t the tip of the sperm, while in others a spiral thickening seems to be present n its surface. Both these conditions are, however, due to a coiling of the distal nd of the sperm body and each represents a stage in the maturation of the perm. The head of a sperm showing the more complex of these two condi-ions is shown in fig. 16, C. Of the two this represents the least mature condi-ion. For convenience this is called stage 1. Stage 2 (the apparently headed perm without a spiral) results from a partial uncoiling of the tip of the sperm (fig. 16, B, D) and a consequent elongation of the sperm body. Further uncoiling of the sperm tip gives rise to the fully mature thread-like sperm. The distribution of these stages is difficult to understand, for completely uncoiled spermatozoa have been found in some specimens to be more abundant than the other stages even in the vas deferens of the male, and have also been found even in the testes. Apart from suggesting that the time and place at which sperms become fully mature is unimportant, this inconsistency remains unexplained. It is, however, presumed that complete uncoiling is necessary before a sperm can penetrate an egg.

The very tightly packed sperms of the vasa deferentia (p. 412) are, in this situation, immobile. Feeble movement is initiated in a small proportion of these when they are released into a 0·6% solution of sodium chloride.

Sperms contained in spermatophores are motile (see above, p. 423), and those in the spermathecae of the female can be seen through the integument to be in constant and active motion, circling round and round within these receptacles. Dissection reveals that these may be in any of the three stages described above. Activity within the spermathecae is maintained for at least 7 days and, if activity and viability are in this case synonymous, probably for much longer.

Sperms are inactivated and presumably killed within a few seconds of being released into fresh water—the medium in which *Dolops* habitually lives. This stands in marked contrast to the behaviour of the sperms of many marine invertebrates when released into sea-water and recalls the maladaptation of the sperms of many freshwater fishes to the medium into which they are released (Huxley, 1930; Rothschild, 1958). One very cogent reason for the always complete protection of sperms from the surrounding water during their transference is thus obvious. Survival of released sperms is greatly prolonged if a 0·6% solution of sodium chloride is used instead of tap-water. Death of sperms released into water is therefore presumably due to the sudden change in osmotic pressure and electrolyte concentration to which they are subjected.

This constant activity of sperms within the seminal fluid stands in marked contrast to the behaviour of those of certain other invertebrates such as sea-urchins in which the spermatozoa are motionless in undiluted semen and only acquire motility, which is intense and of short duration, when shed into water. As the sperms lack any organ of food storage, this constant activity indicates that they are bathed in nutrient fluid.

DISCUSSION

In the spermatophore-producing Crustacea for which functional accounts are available, the wall material is secreted by part of the vas deferens and comes to surround the sperm mass already present. This is so, for instance, in the copepod *Calanus finmarchicus* (Gunnerus) (summary of various papers in Marshall and Orr, 1955), and in various decapods (Spalding, 1942; Mathews, 1954, 1956, and other papers). In *Dolops* the situation is quite different, for spermatophore wall material is produced by glands which are independent of the main reproductive tract, and the only active part played by the vas deferens in spermatophore formation is the injection of sperms into the mass of prospective wall material, which accumulates in special reservoirs. In this respect the process in *Dolops* bears greater similarities to that taking place in certain caddis flies (Khalifa, 1949*b*), cockroaches (Khalifa, 1950), and scorpions (Abd-el-Wahab, 1957; Alexander, 1957, 1959), in which accessory glands are responsible for the secretion of spermatophores, than to that taking place in other Crustacea. (In the light of Alexander's work it is apparent from the detailed account of Abd-el-Wahab that the scorpion with which he dealt produces spermatophores, though he does not specifically state this.) The similarity of the process in *Dolops* and the insects mentioned above is, however, by no means complete even in its grosser aspects, for in most of the insects the wall material (often accompanied by extra protein masses) is ejected before the sperms, which only penetrate it after it has filled the bursa copulatrix of the receiving female. Particularly interesting similarities to the process taking place in *Dolops* are found in certain cockroaches (Khalifa, 1950) in which wall material is secreted into a single sac, into which spermatozoa are injected before spermatophore ejection takes place. The outstanding differences in spermatophore formation here, other than those necessitated by differences in the complexity of the wall, are that only one sac is present in *Blatella* (as compared with two vesicles in *Dolops*) and that the two masses of spermatozoa injected into it maintain their individuality throughout instead of becoming confluent as in *Dolops*.

The spermatophore of *Dolops* is definitely a single structure. In most other spermatophore-producing arthropods in which a double set of organs is involved two spermatophores (as in cyclopoid copepods) or a spermatophoric mass (as in certain decapods) are produced. Spermatophores so produced may fuse, without, however, fusion of the two sperm masses, as in the prawn *Penaeus japonicus* Bate (Tirmizi, 1958), or the alder fly *Sialis lutaria* L. (Khalifa, 1949*b*).

In scorpions, and apparently in pseudoscorpions, a single spermatophore is produced, as in *Dolops*, from paired organs, and its formation in many ways resembles the process which takes place in *Dolops*. Here, however, at least in scorpions, two completed halves are secreted and these do not become confluent but are cemented together by the secretions of special glands (Alexander, 1959).

The discovery of spermatophores in the Branchiura, from which their absence had hitherto been regarded as diagnostic, raises questions regarding the relationships of this enigmatical group. The occurrence of such structures in one genus of the group, however, does little to clarify these relationships, for spermatophores are capricious in occurrence within the animal kingdom and seem to have arisen many times independently in compliance with necessity. For example, while the true fishes as a whole do not employ them they are found in a single isolated case, namely, in *Horaichthys setnai* Kulkharni, and Khalifa (1949*b*) has pointed out that in insects spermatophores have arisen several times in a phylogenetically disconnected manner as a means of solving the problem of sperm transfer. One thing is clear, however: there is no justification whatsoever for suggesting a relationship between the Branchiura and the Copepoda (a group to which the former has frequently been assigned) on the basis of the common possession of spermatophores. Had the two been at all closely related one would expect to find at least some common features relating to spermatophore formation; for although, as Mathews (1954) has shown in the Decapoda, even species of otherwise closely related genera do not necessarily produce similar spermatophores, the process is basically the same within any particular group. In the Copepoda and Branchiura the process is fundamentally different. Thus in the Copepoda spermatophores are formed within modified portions of the main genital tract: in *Dolops* accessory organs are developed. In the Copepoda each spermatophore is from the time of its inception a single structure: in *Dolops* it is of dual origin. In the Copepoda spermatophores are complete before transference: in *Dolops* they are completed only at the time of transfer. After transference they may remain attached to the female throughout life in the Copepoda and in no way interfere with egg-laying, whereas in *Dolops* ecdysis is essential before the laying of fertile eggs is possible. The latter point emphasizes an important distinction between the Copepoda and the Branchiura and one which only Gurney (1949) appears to have stressed: namely, that adult copepods never (or perhaps only as an abnormal and as yet unrecorded event) moult, whereas members of the Branchiura continue to moult throughout life. That an aspect of reproductive biology bound up with the use of spermatophores should involve a relationship to such a fundamental distinction emphasizes the differences between the two groups.

One superficial similarity between the process of spermatophore transfer in *Dolops* and the Copepoda (in so far as one can generalize from the limited observations made on the latter group) is the use by the male in both cases of the legs to assist in spermatophore transfer. In cyclopoid copepods in particular (Hill and Coker, 1930) the process resembles that seen in *Dolops*, and the more precise and well-known method of spermatophore transfer in at least some calanoids presumably had a similar origin. Such similarities are, however, of as little value as the possession of spermatophores in deciding affinities, for it is apparent that whatever appendages are most conveniently situated for this purpose will be used to guide the spermatophore into position. Thus in cyclopoid copepods several pairs of legs play a part in spermatophore transfer, whereas in calanoids only the fifth pair, which is rudimentary in cyclopoids, is concerned in spermatophore transfer. Similarly in certain decapods, presumably because of the location of the male genital aperture, the abdominal appendages assist in spermatophore transfer.

On the other hand, the possession of spermatophores by *Dolops* gives no indication of any affinities between the Branchiura and the Branchiopoda, in which spermatophores are completely unknown. The evidence therefore tends o emphasize the isolated nature of the Branchiura rather than to indicate ffinity with any other group.

Similarly a study of spermatophores, beyond emphasizing the isolated osition of the genus *Dolops*, throws little light on the relationship to each ther of the various branchiuran genera.

As to how the evolution of spermatophores could have taken place, the ollowing suggestions are offered. In at least some species of *Argulus*, and robably in all, accessory glands homologous with but much less extensive han the spermatophore glands and spermatophoric canals of *Dolops* are resent, and presumably represent an anatomical feature of long standing in he Branchiura. According to the figure given by Wilson (1902) for *A. ameri-anus* Wilson, and to my own observations on *A. africanus* Thiele, these rgans take the form of rather short, blind ducts with no great glandular fflorescence distally. It may be that in the primitive argulids, which would nclude the ancestors of *Dolops*, they secreted some kind of cement which ormed a temporary 'seal' around the opposed genital apertures of the male nd female during copulation. On the evolutionary line taken by *Argulus*, eading to the development of complex claspers on the legs of the males, in hich, in certain species at least (personal observations), a conical papilla with wide aperture has been developed to serve as a primitive penis, there would e no need for the production of this secretion to be copious and therefore no endency towards spermatophore formation. The more copious production f such a secretion in forms less specialized in these directions would tend owards the formation of a bubble at times of copulation and this would, uring its transient existence, afford protection to the sperms during trans-erence. It would require but a small step to make the bubble a more permanent feature of the reproductive process. Its gradual modification and the tilization of the spermathecal spines already present (as must have been he case in order to allow of perforation of the egg-membranes) would lead to he kind of structure seen today. Throughout this process selection would avour the production of more abundant and more suitable cement, and he spermatophore gland and its associated canal and reservoir would radually increase in size and specialization towards this end. The carapace obes, filled as they are with parenchymatous cells and having abundant aemocoelic spaces, would readily permit the incursion within them of landular tissue, in much the same way as they came to house the digestive iverticula of the alimentary canal.

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The Early Post-moult Cuticle in *Buthus*

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SUMMARY

The cuticle of the scorpion, *Buthus tamulus*, has been studied at the early post-noult stage. There is a distinct two-layered epicuticle, which is non-chitinous, and procuticle consisting of a chitin/protein complex. Polyphenols, phenolase, and ulphur are present at this stage. Quinones and groups containing sulphur seem to lay a part in the subsequent hardening of the cuticle.

INTRODUCTION

SINCE Krishnan (1953) reported the occurrence of —S—S— bonds in the cuticle of *Palamnaeus swammerdami*, a keen interest has been evinced in the cuticle of arachnids in general. Even earlier, Lafon (1943) noted the presence of over 2·85% of sulphur in the cuticle of *Limulus* and suggested that the cuticular proteins were similar in this respect to the keratinous type of proteins of the vertebrates. On another count, the cuticle of arachnids attracted the attention of workers in the field. Browning (1942) reported that what was considered to be the epicuticle in the spider, *Tegenaria atrica*, was only an optical artifact. Although later work on the same genus by Sewell (1955) showed the presence of an epicuticle, the homology of the arachnid epicuticle was once again a bone of contention; for Krishnan, Ramachandran, and Santanam (1955), studying the X-ray diffraction patterns of the epicuticle in *Palamnaeus*, observed the presence of chitin in this layer. Until then the epicuticle was considered to be a non-chitinous lipoprotein layer, distinct from a lamellated chitin/protein complex constituting a procuticle. Kennaugh (1959), investigating the cuticles of *Pandinus imperator* and *Scorpiops hardwickii*, reported that the epicuticle in these forms is free from chitin. What Krishnan described as the inner epicuticle corresponds to what Kennaugh called hyaline exocuticle. Shrivastava (1954) did not find any sulphur in the inter-moult cuticles of *Palamnaeus bengalensis* and *Buthus*. And again, on the basis of the amino-acid constitution of the cuticular proteins, Hughes (1959) suggested the possibility of the occurrence of quinone/sulphur links in an acarine.

Thus the questions whether there was a chitin-free epicuticle and whether sulphur played a part in hardening the cuticle remained unsolved. Hence it was considered desirable to study the histochemistry of the newly exposed, soft, early post-moult cuticle of a scorpion.

MATERIALS AND METHODS

The colourless soft cuticle from the freshly moulted scorpion, *B. tamulus*, has been used for the present study. Fresh hand-sections, as well as frozen

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and paraffin sections, were made and studied in both unstained and stained preparations. Mallory's triple, Masson's trichrome, and Lower's iodophil stains have been used to colour the cuticle.

Xanthoproteic, Millon's, Baker's, Sullivan's, Jackson's, Schulze's, Nadi, azide/iodine, and chitosan tests were amongst the few histochemical tests employed.

OBSERVATIONS

Structure and staining reactions

In the newly moulted scorpion the cuticle appears opaque white when it is in contact with the underlying tissues, while on separation and cleaning it is seen to be colourless and transparent. In surface view it presents a granulated appearance except where the tonofibrillae are attached to it. In the latter places the surface presents a honeycomb pattern. The ducts of the tegumental glands appear as rounded openings, distributed all over the body. The area around the opening of the ducts does not appear different from the rest of the surface.

In cross-section the cuticle appears as a thin layer 12 μ in width. This thin strip is composed of two distinct divisions: an outer non-lamellated, homogeneous layer, 3·5 μ thick, and an inner lamellated layer, 8·5 μ thick. In regions where the cuticular surface is granulated, the outer layer appears sinuous because of the thickening. When sections are stained by Masson's or Mallory's method, the two layers are more prominently distinguishable because of their differential staining. The outer layer stained red with both the stains while the inner (lamellated) layer stained blue with Mallory's and green with Masson's stain. In accordance with the nomenclature suggested by Richards (1951), these two layers may be termed the epicuticle and procuticle. A very thin layer at the outer border of the epicuticle stains differently from the rest of the outer layer. This thin border stains blue with Mallory and green with Masson. These two portions of the epicuticle may be termed the inner and outer epicuticle, corresponding to those of the larva of *Sarcophaga* (Dennell, 1946). No subdivisions of the procuticle are seen at this stage, although in the inter-moult cuticle there may be as many as three distinct subdivisions.

Chemical nature

Gross studies of the chemical nature of the newly moulted cuticle indicate the presence of fundamental differences in the chemical composition of the two layers (epicuticle and procuticle). Treatment with hot saturated alkali dissolves the epicuticle, while the procuticle is resistant to the treatment. The procuticle after this treatment colours violet with iodine / sulphuric acid, and dissolves in 3% acetic acid; this suggests the presence of chitosan. Treatment with concentrated sulphuric acid dissolved both the layers, but the epicuticle was more resistant than the procuticle.

The proteins of the cuticle, as indicated by the xanthoproteic and biuret reactions, are distributed throughout the cuticle, although there seem to be differences in the intensity of the reaction in the two layers. With the xanthoproteic test the epicuticle was more intensely coloured than the procuticle, while with Millon's reagent the cuticle was only faintly positive (see table 1).

TABLE 1

Summary of the reactions of the early post-moult cuticle

Reagent	*Epicuticle*	*Procuticle*
Xanthoproteic	+++	++
Millon's	+	+
Millon's (Baker's modification)	++	++
Chitosan	−	++
Jackson's	+	−
Sudan black B	+	−
Argentaffin	+	+
Nadi	+	−*
Nadi with KCN	−	−
Guaiacol and phenolphthalein	blue	colourless
Mallory's stain	blue† red‡	blue
Masson's stain	green† red‡	green

* The procuticle is also coloured royal blue, but this was considered to be due to diffusion as it is faint and appears after a lapse of time.

† Outer epicuticle.

‡ Inner epicuticle.

The table shows that there are sudanophil substances in the epicuticle but not in the procuticle. However, the surface of the cuticle does not show any colouring of lipids, either immediately after moulting or within the next 12 h. This fact presumably indicates the absence of a separate paraffin layer similar to the one described in *Rhodnius* (Wigglesworth, 1947).

Aromatic substances in the cuticle are of considerable importance because of their role in the tanning of the cuticle. Their importance in the insect cuticles has been demonstrated by several workers (Pryor, 1940; Dennell, 1947*a*). It was shown that the cuticle in an arachnid *Thelyphonus* is hardened by phenols (Krishnakumaran, 1959). The role of phenolic substances in sulphur-hardened cuticle is obscure.

In the early post-moult cuticle of *Buthus* Millon's test is only faintly positive. However, Baker's test (mercuric sulphate and sodium nitrite) and the argentaffin reaction are strongly positive in the cuticle and also in the ducts of the tegumental glands. The formaldehyde / sulphuric acid test and the phosphomolybdic acid test for polyphenols (Feigl, 1954) are positive. Another significant feature of the cuticle at this stage is the strong Nadi reaction, which is cyanide-sensitive and thermolabile. The colour reaction with guaiacol and phenolphthalein indicates that the enzyme involved is a phenolase.

In addition to the phenolic substances and also the phenolase, there are some sulphur-containing compounds in the cuticle. The azide/iodine reaction, by immediate ebullition, suggests that the sulphur is in the form of sulphydryl or thiosulphate or sulphide. Since pretreatment of the cuticle with dilute mineral acids does not reduce the reaction with the azide/iodine, it is presumable that thiosulphates and sulphides are not involved. Pretreatment with iodine solution, however, renders the cuticle less sensitive to the action of the azide/iodine reagent. This is presumably due to the oxidation of —SH groups to —S—S— groups, and the latter do not react easily with the azide/iodine reagent (see Feigl, 1954). The nitroprusside and ferricyanide reactions are positive in fresh material. The distribution of sulphur-containing compounds was not clear, as the cuticle is very thin and the region from which nitrogen evolved could not be determined accurately. For the other two reactions hand-sections were used, and these were too thick to show definitely whether the positive reaction was in the epicuticle or in the procuticle. However, it is clear that the sulphur is present in the procuticle.

Changes after moulting

The changes that the cuticle undergoes soon after the moult involve the argentaffin and the sulphur-containing substances. Changes in the colour and the staining properties are also noticeable. Twelve hours after the moult the cuticle is reddish yellow, and in sections the lamellation previously noted in the procuticle is to a large extent masked. When these are stained the entire procuticle becomes red with both Mallory and Masson. Thus the procuticle is impregnated with some acidophil substances, presumably the tanning precursors. There is thus a correspondence with the stage described in the cuticle of *Schistocerca* (Schatz, 1952). The layer concerned may be termed the mesocuticle (see Lower, 1956).

The chemical nature of the procuticle at this stage shows that the procuticle is also positive to Sudan colouring agents and that the xanthoproteic reaction is equally strong in the epicuticle and the subjacent procuticle. The Nadi reaction is not thermolabile and is insensitive to cyanide. The argentaffin reaction and also the azide/iodine reaction are not as strong as in the early post-moult stage. The azide/iodine reaction is hastened by treatment of the cuticle with alkaline ferrous hydroxide solution. Corresponding with these changes in the chemical constitution, the cuticle becomes more resistant to the action of acids and alkalis. The surface of the cuticle is no longer wettable and appears to be stretched taut.

DISCUSSION

From the foregoing observations it is evident that the cuticle of *Buthus* is fundamentally similar to that of the insects and other arthropods that have been investigated. As in the other arthropods, it is broadly distinguishable into a non-chitinous, lipoproteic, structureless, homogeneous, thin epicuticle and an underlying, broader, lamellated procuticle, formed of a chitin/protein complex. The latter seems to differentiate into subdivisions which have been described in the inter-moult cuticle of this and other scorpions. Evidence in favour of the view that the epicuticle is a double-layered, non-chitinous region has been obtained in the present investigation of the unhardened cuticle of *Buthus*. At this stage, the epicuticle is not resistant to the action of hot aturated sodium hydroxide solution, and this suggests that it is not chitinous. It is also suggested that the great resistance of the inter-moult epicuticle is due to the hardening of this layer, rather than to the presence of chitin nextricably bound with protein (see Krishnan, 1956). Any chitin found in he epicuticle of the inter-moult cuticle should have passed into this layer after it is deposited; but such a deposition of chitin is considered unlikely.

The positive Nadi reaction in the newly formed cuticle, its sensitivity to heat and to cyanide, and the colour reaction with guaiacol and phenolphthalein ndicate the occurrence of a phenolase in the cuticle at this stage. It has been shown that such an enzyme as this, in the cuticle of insects and Crustacea, is responsible for the conversion of polyphenols into quinones (Dennell, 1947 *a*, *b*). The positive reaction to Baker's modification of Millon's test, to the formaldehyde / sulphuric acid test, and to the phosphomolybdic acid test indicates the presence of phenolic substances, presumably the precursors of tanning agents.

The presence of sulphydryl groups in the early post-moult cuticle is a noteworthy feature. Their absence later, owing presumably to their conversion to —S—S— groups, is significant. (The slow azide/iodine reaction in freshly-formed cuticle, together with the hastening of the reaction by treatment with alkaline ferrous hydroxide solution, suggests the presence of disulphide or similar groups (Feigl, 1954).) As no specific enzyme is involved in the oxidation of —SH into —S—S— groups, it is here suggested that quinones may play a significant part in this conversion. Whatever may be the mode of formation, the quinones and the disulphide groups appear at about the same time. (The cyanide-insensitive Nadi reaction and the faint colour of the cuticle suggest the presence of quinones.) By about the same time the changes in the staining properties and the resistance to the action of acids and alkalis are noticeable. All these indicate that the cuticle is preparing for the tanning and is probably already tanned to some extent.

Hughes (1959), in his study of the cuticle of a tick, suggested that the quinones and sulphur are linked together. The basis for this suggestion was a high percentage of lysine in the cuticular protein, which rendered quinone tanning unlikely. Krishnakumaran (1959) suggested the presence of similar bonds in the cuticle of *Galeodes*. On the basis of the circumstantial evidence, it is here suggested that the sulphydryl groups are converted into disulphide groups by the wave of quinones formed by the oxidation of phenols. This simultaneous formation of quinones and disulphide groups aids the condensation of both these groups with the protein to form the resistant material that seems to be characteristic of the cuticles of arachnids.

The absence of any paraffins (i.e. sudanophil material) on the surface of the cuticle indicates that there is no wax layer comparable to that of *Rhodnius*; and the loss of 'wettability' of the surface in cuticles at the 12-h stage is to be correlated with the hardening of the epicuticle.

Thus, while the cuticle of *B. tamulus* resembles that of other arthropods i having a chitin-free, double-layered epicuticle and a procuticle composed o a chitin/protein complex, hardened in part by phenols, it differs from ther in the occurrence of sulphur-containing compounds which also seem to pla some part in the hardening.

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A Contribution to the Life-history of the Brachiopod *Tegulorhynchia nigricans*

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Summary

This study of the development of a rhynchonellid brachiopod has revealed marked differences from that of *Terebratella inconspicua* (Percival, 1944). These principally concern the origin of the mesoderm and of the lophophore. The origin of the right and left mesoblastic sacs in *Tegulorhynchia* and in *Terebratella* depends on the stage at which the blastoporal groove exerts its influence, earlier in the former, leading *directly* to their formation, later in the latter, leading *indirectly* to their formation. The subsequent division into anterior and posterior sacs is similar in both genera. The lophophore of *Tegulorhynchia* is the product of the anterior lobe, and considerable centripetal movement occurs during the sinking of the centre from which the brachial fold (or buccal lip) is derived. The process of formation of the lophophore in *Terebratella* can be regarded as a much shortened version of that which occurs in *Tegulorhynchia*. In the latter genus the origin of the brachial fold is elaborate and protracted, while in the former the brachial fold does not appear until 8 pairs of cirri have been formed.

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Habitat

TEGULORHYNCHIA NIGRICANS Sowerby is abundant in a rock pool in Lyttelton Harbour, where conditions favour its occurrence in numbers rarely encountered by the shore collector. The pool stands full of water at half-tide and is throughout accessible by wading. Boulders of varying sizes lie singly or in groups, some completely and continually submerged, others partly exposed by the receding water. Many boulders rest one on another and provide crevices through which water seems to pass slowly and without violence, creating no disturbance in the vicinity. The superimposed boulders form a group to one side, near a rocky platform, some half to three-quarters of a metre higher than water level at half-tide, and when tidal flow occurs water enters the pool round the two ends of the platform without causing undue disturbance in the pool.

Abundant vegetation in the pool includes *Hormosira*, *Blossevillea*, *Corallina*, occasional plants of *Macrocystis*, and in summer and autumn *Rama novae-zelandiae*.

Tegulorhynchia nigricans and *Terebratella inconspicua* Sowerby, the latter

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also abundant, are conspicuous on the continuously submerged boulders, and are usually separated into rather distinct populations, although there is some mixing. A third small brachiopod, *Pumilus antiquatus* Atkins, is present, variously distributed among the two larger forms.

The uppermost of the submerged superimposed boulders often have beneath them from a few to very many *Terebratella*, perhaps several score. The deeper stones may carry far more *Tegulorhynchia* than *Terebratella*. Where the two exist on the same stone, *Tegulorhynchia* usually occupies the central position, at any rate that which seems least disturbed. Further, *Terebratella* is not uncommonly found among *Lithothamnion*, while *Tegulorhynchia* rarely occurs in such a place.

In this pool a deposit of very fine material normally rests on the brachiopods as elsewhere. It consists almost entirely of organic matter; there remains only a trace of ash on ignition. This itself indicates very quiet conditions among the stones. There is usually a thicker deposit on *Tegulorhynchia* than on *Terebratella*. 'Silty' deposit is absent from the rare specimens carrying *Lithothamnion*.

The shells of the rhynchonellids usually carry a variety of animals, such as Foraminifera, sponges, hydroids, various polychaetes, nemertines, Polyzoa, young molluscs, ophiuroids, mites, pycnogonids, copepods, ostracods; in fact, the surfaces of these valves carry little worlds where many small forms live for quite a time. The corrugated surfaces collect the fine mud which forms a suitable medium for many of the errant animals. This is usually not so with *Terebratella*, the shell of which is smooth, does not carry so much deposit, and, for some reason, seems less attractive to small animals than is the other. This dissimilarity is shown in the way that brachiopod larvae settle. While individual shells of *Tegulorhynchia* may carry scores of newly settled *Terebratella*, and, later, of *Tegulorhynchia* and *Pumilus*, it is rare that shells of adjacent *Terebratella* are found to carry newly settled brachiopods.

This apparent discrimination by the larvae has been seen in the laboratory when live specimens of the two species have been offered for settlement.

The connexion between quiet conditions and large populations of brachiopods has been emphasized by finding, in other places on Banks's Peninsula, pools subjected to strong wave action and having large boulders movable by at least two men. Such stones have carried very few, perhaps two or three, *Tegulorhynchia* on the sheltered side. *Terebratella* has been found in similar conditions in the tidal zone, sometimes on the same stone as carried the other species.

BREEDING SEASON

Reproductive individuals have been found from 2 May to 26 June, a period which overlaps the breeding season of *Terebratella inconspicua* at the other side of the Harbour (Percival, 1944). In the pool at present under consideration, *Terebratella* breeds rather later than do its relatives mentioned above, but finishes before the end of the season of *Tegulorhynchia*.

The smallest reproductive individuals of *Tegulorhynchia* found have been a female, 9 mm by 9 mm, and a male, 8·5 mm by 8·5 mm, out of a range from newly-settled forms to adults 2 cm in diameter.

The sex ratio of samples varies considerably from 1:1 according to the sample. Sometimes males preponderate, sometimes females. As many as 14% of individuals above the minimum size at maturity may be sterile.

Egg-production varies somewhat, independently of size of individual; for instance, the following numbers of eggs were found in relation to shell diameter: 8,680 eggs, 2 cm diameter; 4,440 eggs, 1·75 cm diameter; 5,260 eggs, 1·60 cm diameter; 4,080 eggs, 1·40 cm diameter.

The eggs are liberated into the mantle cavity and held in the lophophore by the bending of the cirri towards its apex, a basket being formed, from between the bars of which the young cannot escape unless the cirri uncurl. What causes the final escape of the mature larvae has yet to be determined.

Spermatozoa are shed into the surrounding water and spread through a colony. Chances of effective insemination are probably better in a larger population than in a smaller. Sometimes all the individuals in a group of 3 or 4 may be of the same sex.

DEVELOPMENT

Eggs obtained by tearing open ripe ovaries float out freely. Each has a follicle, more or less complete, of cells about 6 times as high as the thickness of the egg cellwall. On artificial insemination, the cells of this layer double in thickness and change from a flattened form to a hemispherical, closely packed condition. The layer meanwhile breaks, perhaps through slight swelling of the egg or by shrinkage of the follicular layer. During the process, the egg alters in shape from roughly discoidal, or polygonal, or irregular, to spherical. The cells are later sloughed and the egg-wall is then naked.

Some dimensions are: newly fertilized egg, 0·16 mm in diameter; gastrula, 0·17 mm in diameter; late larva, length, 0·2 mm, greatest width, 0·14 mm, with setae 0·15 mm long.

Cleavage. It is usual for cells to be of equal size after each division, although naturally-spawned eggs may produce cells slightly different in size. Since in other phyla so many cases occur of size-differences between blastomeres, particular attention has been paid to conditions in the present case, and here, as was noted in *Terebratella*, the normal condition is considered to be uniformity of size of the early blastomeres.

Artificial insemination has led to a variety of divisions, from equal to very unequal; for instance, a 4-cell stage gave two undivided cells and a mass of about 20 micromeres; death usually took place early.

Commonly, in nature, the first division is slightly oblique, while the second division produces two cells touching at one pole and two others touching at the other pole. Sometimes division is radial, producing 4 cells touching in the same plane and having an axial cavity, all these in the same brood. The oblique and radial cleavages lead finally to a spherical blastula at the 8-cell stage.

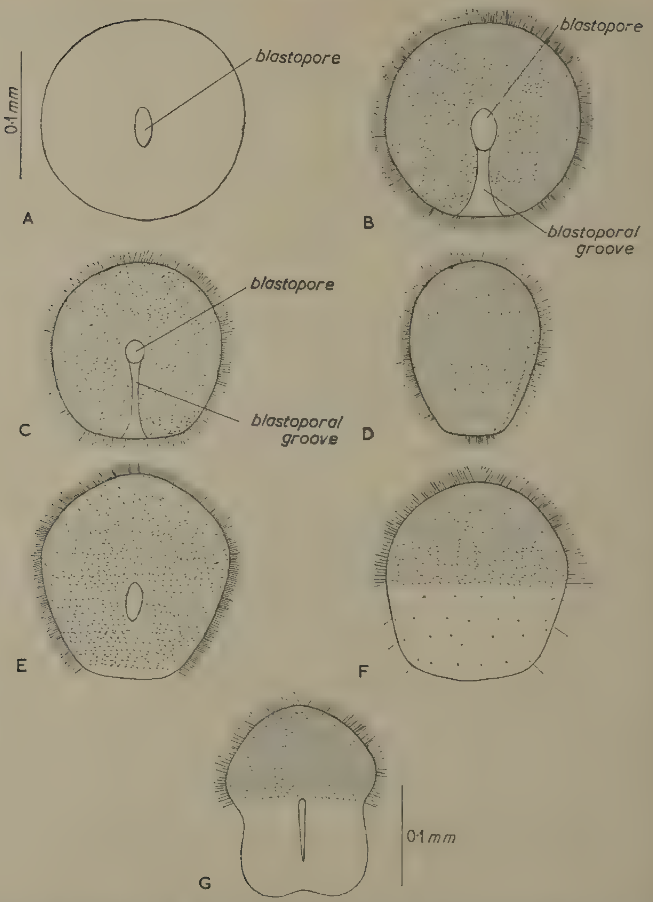


FIG. 1. A, early gastrula with blastopore closing. B, gastrula with blastoporal groove. C, later gastrula with deepening groove. D, same as C, but from the side, showing the blastoporal groove. E, late gastrula becoming pentagonal and losing posterior cilia. F, abblastoporal view of later gastrula with posterior cilia nearly all gone. G, pentagon with late blastoporal groove and beginning of body differentiation.

Gastrulation. Invagination gives rise to a gastrula, the epiblast becoming ciliated early. The blastopore changes from circular to elliptical with the long axis in the future antero-posterior direction (fig. 1, A). Soon it sinks slightly into a wide recess which then continues backward as a slight groove (fig. 1, B). The future posterior part becomes slightly flattened dorso-ventrally, the beginning of a change in outline after gastrulation.

Movement at this stage may be in any direction, with the blastoporal or the abblastoporal surface upward. Later, the tendency of movement is toward the future anterior side.

Embryogenesis. The external changes are to some extent similar to those described in *Terebratella* (Percival, 1944). The late gastrula becomes bluntly wedge-shaped, with a well-defined blastoporal groove passing to the presumptive posterior margin (fig. 1, C, D). The head of the wedge is anterior, bluntly rounded, the rest of the body tapering backwards on all sides from about the middle of the blastopore, more on the blastoporal and abblastoporal sides.

During the assumption of the blunt wedge-shape, cilia disappear from the flattened posterior end (fig. 1, E), the loss continuing forward to the transverse plane passing through the anterior part of the blastopore, so that ultimately the rounded end is ciliated, and the rather squarish posterior end is non-ciliated. The ciliated portion becomes the anterior lobe from which the lophophore and the stomodaeum will be produced, while the unciliated part becomes the mantle and the pedicle.

The body becomes somewhat pentagonal in plan (fig. 1, F) and, when viewed closely at this stage, the anterior ciliary movement is seen to divide at the apex into two lateral waves passing outwards. Gradually, the pentagonal shape is more clearly defined and the blastoporal groove narrows (fig. 1, G). The blastopore is reduced in size from behind forward until it is a very small pit leading into the enteric cavity. During this time, as will be seen later, the mesoblastic sacs are being cut off from the archenteron; the hind end is slightly bilobed.

Change of shape leads to a body with a distinct, ciliated, anterior lobe, bluntly and broadly conical, and a squarish posterior portion, rather narrower than the base of the anterior lobe, thinner than broad. The vestige of the blastopore is in the base of the anterior lobe, and the remnant of the blastoporal groove lies in the mid-line of the posterior portion.

Beecher (1891) has collected the terms that have been applied by various authors to embryonic and larval phases of brachiopods and are used here in the descriptions of the figures.

In *Tegulorhynchia*, the marking off of the anterior lobe as a broad, blunt, ciliated region and of the posterior portion as a bluntly rounded, slightly bilobed mass leads to the first appearance of the mantle rudiment (fig. 2, A). This begins as a constriction behind the widest, posterior, part of the anterior lobe, localizing the cilia on that mass. Next behind the constriction appears a transverse ridge, on the abblastoporal or dorsal side, the ends of which grow downwards and then towards each other in the mid-ventral or blastoporal line, so that, finally, the body consists of a ciliated anterior lobe, an unciliated transversely continuous mantle rudiment in the form of a ridge higher dorsally than ventrally, and a posterior, unciliated, pedicle rudiment (fig. 2, B). The blastopore has meanwhile closed, and the blastoporal groove has disappeared.

About the time of the appearance of the abblastoporal or dorsal mantle

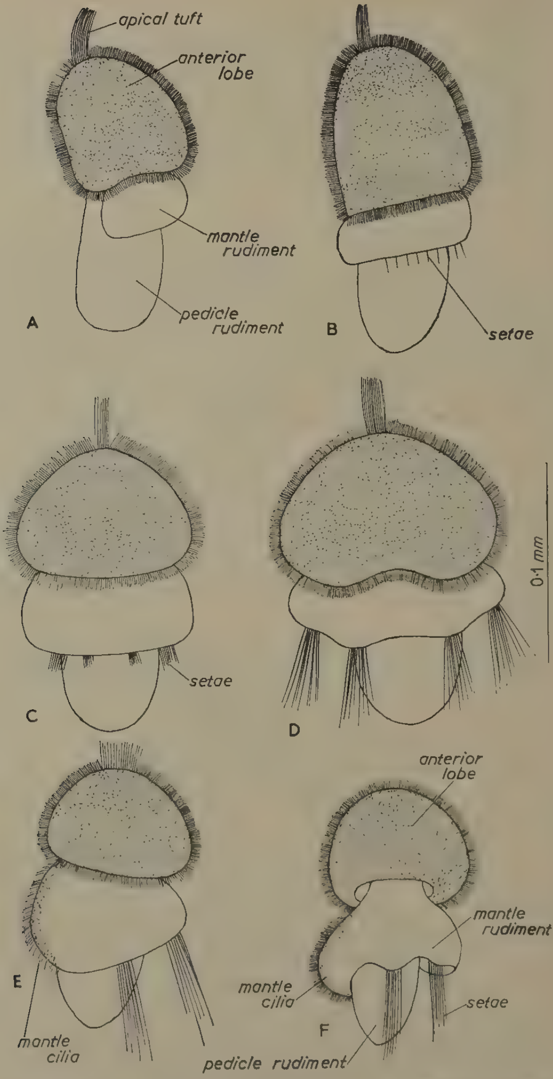


FIG. 2. A, beginning of regional differentiation; anterior lobe with apical tuft, dorsal and lateral mantle ridge, pedicle. B, complete mantle ridge, early setae, apical tuft. C, extending mantle fold and elongating setae. D, more advanced larvae. E, side view of later larva. F, mature larva, from side; no apical tuft.

rudiment, an apical tuft of long cilia appears rather quickly and is a distinctive feature for some time (fig. 2, A).

When the mantle rudiment surrounds the body, short setae appear dorsally and laterally on its posterior border. They grow longer and soon show as two pairs of tufts of long setae, a pair of dorso-lateral tufts and a pair of lateral tufts, which readily enable the orientation of the specimen to be determined (Percival, 1953 (fig. 2, C, D).

The mantle rudiment extends backwards as a sheath over the pedicle rudiment. Its posterior margin becomes lobed and sinuous, each lobe carrying a tuft of setae (fig. 2, D). Evidence of the presence of muscles is later given by the ability of the setae to be raised and lowered while larvae are swimming. Whether the setae are important as flotation organs is not very clear; the larvae have not been taken in plankton collections. The larva of *Terebratella inconspicua*, on the contrary, has been taken in a plankton collection near the surface; its setae are extremely minute and appear vestigial. The complete mantle rudiment of the larva of *Tegulorhynchia* extends about half-way along the pedicle rudiment, never lengthwise ensheathing it, as does the mantle rudiment of *Terebratella*.

Late larvae, liberated from the parent, show on the ventral side of the mantle rudiment a longitudinal, ciliated band by means of which the body can be propelled when applied to the substrate (fig. 2, E, F). What part this plays in open-water locomotion is not clear.

With further differentiation, the apical lobe becomes distinctly separated from the rest of the body by a deep constriction which forms a tapering neck widening into the mantle. This neck is highly mobile and can be lengthened and shortened and bent one way and another, indicating the presence of muscular tissue inside.

Live specimens stained with neutral red take the stain densely in the apical lobe, round the posterior margin of which may be seen about 48 well-stained, ruby-coloured spots (? eyespots) capable of being followed for some time during metamorphosis.

The beat of the cilia on the apical lobe varies from place to place. Particles are driven in a clockwise direction by the cilia of the hemispherical apex. The cilia of the posterior region drive particles outwards and backwards, while a band between the two areas causes particles to be drawn in and immediately shot out. Cilia on the ventral face of the mantle rudiment drive particles backwards.

Earlier pentagonal embryos travel forward on the long axis with an anticlockwise spin. The course also gyrates anticlockwise. Later, the axial spin is very much reduced and ultimately usually absent, while the gyration remains.

Some time before liberation of the larvae by the parent, the apical tuft of cilia disappears, the setae are well developed, and the animal is ready for metamorphosis (fig. 2, F). Artificially liberated larvae may swim or creep for a week or more before any sign of settlement and metamorphosis appears.

Metamorphosis. The time spent between natural liberation and settlement varies somewhat. It is usually much longer than that taken by *Terebratella*, which ranged from 24 to 36 h. The shortest settling time for larvae of *Tegulorhynchia* was 48 h, although specimens in the same brood continued swimming for several days, and indeed for as much as a week, before settlement. By no means all settle in the laboratory; very many die, presumably from exhaustion.

What induces settlement is not yet known (see p. 454). Larvae will not settle on newly submerged stones which have been dry for weeks, nor on clean, new glass. They have settled on a stone and on glass which have been exposed for a few days to sea-water, but have not completed metamorphosis. In the latter case, changes took place internally and externally which normally proceeded early in metamorphosis, but it seems that they began to lose step, and monsters arose which died.

Normal changes occur when larvae settle on live *Tegulorhynchia* shells or on stones freshly taken from the natural environment. After creeping about horizontally, propelled by the ventral cilia of the mantle rudiment, they stand on the tip of the stalk and attach themselves by means of a secretion from the end of the pedicle. The mantle shortens and thickens, forming a conspicuous belt round the body, while the setae swing upwards and project in fan-like tufts horizontally (fig. 3, A). Gradually the setae tilt upwards with an upward rolling of the mantle edge, until they project upwards and outwards. The process is similar to that described in *Terebratella* (Percival, 1944, p. 9).

During this time, longitudinal contraction has pulled the apical lobe inward to broaden it and to change the surface into a low central mound surrounded by a broad margin on the edge of which lie the 'eyespots'. The low central mound gradually sinks inward, and a crescentic slit appears between it and the broad margin on the dorsal side. The sinking continues, and another crescentic slit appears opposite, between the mound and the margin (fig. 3, B). The two crescentic slits later become confluent, separating a central boss from a broad peripheral ring. There is evidence that the central mass is being pulled in and that the peripheral band gradually decreases its diameter for a time. In specimens stained during life with neutral red, the brightly coloured 'eyespots' move inward from the edge of the peripheral band to half-way between the outer and inner edges, when they lose the stain and are no longer visible. Meanwhile, the central mass has sunken considerably and the two crescentic slits mentioned above have united, forming a circular slit, and have cut off externally the central from the peripheral area. It is to be noted that neutral red disappears from the apical mass by the time that these changes have occurred.

During these changes in the apical lobe, the mantle rudiment has been moving upwards, and the setae swing round through nearly 180°. The follicles of the setae show very brightly when stained with neutral red.

While there are still two crescentic slits in the apical lobe and the mantle

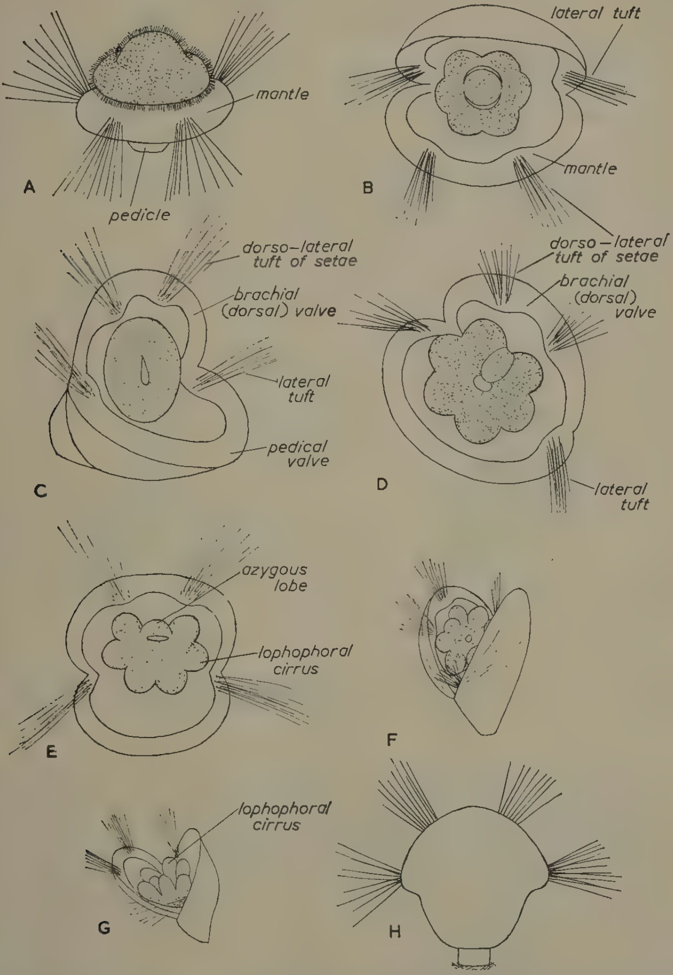


FIG. 3. A, early settlement with half-reversal of mantle and broadened, retracted, apical lobe. B, full reversal, anterior and posterior invaginations cutting off boss of apical lobe before its withdrawal; valves clearly differentiated; rudiment of 6 cirri. C, boss of apical lobe withdrawn; no rudiment of cirri showing. D, stomodaeum; movement of brachial lip rudiment from pit towards gap in horseshoe. E, well-developed cirri; brachial lip placed in ring of cirri. F, later stage showing early lophophore. G, another drawing of the same. H, young adult, dorsal side up.

margin is still a circular thickening, the new outer surface of the mantle begins to secrete the initial shell, which is bivalvular and extremely thin. The mantle is not bivalvular, but tubular, until the two crescentic slits have joined; then the mantle edge changes to a ventral pedicle edge and a dorsal or brachial edge. Flattening dorso-ventrally next occurs, the dorso-lateral tufts of setae being on the edge of the brachial valve and the lateral tufts of setae being on the pedicle valve, very close to the angles of the gape.

The new-transformed apical lobe lies as a short, thick tongue contained in a bivalve sheath, supported on a short, rather thick stalk.

Origin of the lophophore. While the central mound of the apical lobe sinks from sight (fig. 3, c), the thick margin becomes broken by a gap on the dorsal (brachial) side, forming a horseshoe-shaped, thick rim with the two ends touching (fig. 3, D). The first crescentic slit appeared in the radius of the gap, and is still, in life, deeper than any other part of the circular slit. The outer edge of the horseshoe band becomes hexagonal.

About this time, an azygous lobe appears on the brachial side of the central pit into which the central area sank, and moves into the gap between the ends of the horseshoe-shaped rim, projecting towards the centre of the pit. The pit, now apparent, becomes the gullet and is formed from the second crescentic slit. The azygous lobe is the reappearing central mass of the anterior lobe and will form the buccal lip or the brachial fold (fig. 3, D). The horseshoe-shaped rim, faintly hexagonal, will become the cirrous part of the lophophore, the 6 angles being the rudiments of the first 6 cirri (fig. 3, E-G).

The first 6 cirri are arranged two on the side of the pedicle, two laterally, and two on the brachial side, separated by the azygous lobe or buccal lip rudiment.

From what has been indicated by means of the stained 'eyespots', the lophophoral cirri are derived from the base of the apical lobe, which has been rolling upwards centripetally toward the apex since settlement.

During these changes, the apical lobe has projected towards the mouth of the mantle cavity, visible when the animal has been undisturbed, but enclosed on disturbance. Soon, however, the modified apical lobe gradually shortens, flattens dorso-ventrally, and spreads itself on the inner face of the brachial valve, the lip or azygous lobe being outermost (compare with *Terebratella*). The apical lobe thus transforms into an oral field consisting of a shallow basin (outer stomodaeum) bordered by the 6 cirri and the buccal lip rudiment. The stomodaeum has not yet opened into the mid-gut rudiment. The oral field will be elaborated into the lophophore.

The position of the oral field on the inside of the brachial valve is such that when the valves close the oral basin or pit or outer stomodaeum becomes narrow and slit-like, with its long axis lying transversely. The 6 cirri lie longitudinally, tips towards the free edges of the valves.

With growth, the cirri elongate and, in an open mantle cavity, form a cylindrical, ciliated tube leading to the gullet, the brachial side broken by the buccal lip. The lip changes from a lobe to a rather thin flange or hood projecting slightly toward the middle of the oral field which is surrounded by the 6 cirri.

Growth of the whole body proceeds for some time before the cirri increase in number.

Additional cirri are formed simultaneously at the two ends of the cirrous arc, at each side of the buccal lip, and come to lie immediately on the convex margin.

After the formation of 8 pairs of cirri, the buccal lip enlarges towards the cirri, forming a crescentic slit leading into the gullet. On the brachial side is the lip, derived from the central area of the apical lobe; on the pedicle side is the crescent of cirri derived from the margin beyond the 'eyespots' of the apical lobe. Thus the rudiment of the lophophore is established (fig. 3, G), and the juvenile mouth is transformed into the adult food-groove and mouth; from the first, the lophophore is zygomorphic.

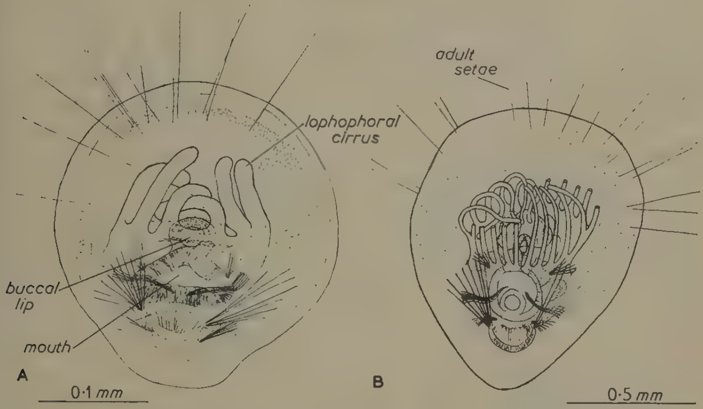


FIG. 4. A, advanced young adult, still with only 6 cirri and brachial lip above the mouth; note muscles. B, advanced young adult with 10 pairs of cirri and a well-developed brachial lip.

The food groove or buccal groove grows during the addition of the cirri on each side of the lip, so that right and left elongations are formed of the tip, and the cirrous ridge produces the arms of the lophophore. Lengthening of the arms is accommodated by the elaboration of the spirolophe and by the increase in the depth of the valves, especially the brachial, as well as by increase in length and breadth. During this period also the setae have changed in distribution from tufts to fringes (figs. 3, H; 4, A, B).

Internal changes before metamorphosis. The origin of the mesoderm is heralded externally by the formation of the blastoporal groove and the introduction of the wedge shape of the body (fig. 5, A). The groove deepens and lengthens somewhat so that its floor presses inward towards the opposite, abblastoporal ectoderm, which it meets, and cuts the posterior part of the archenteron into two limbs, one on each side of the newly formed longitudinal, ectodermal partition. Thus, momentarily and ideally, the archenteron consists of an anterior sac, into which opens the blastopore, and two postero-lateral sacs or enterocoeles. However, one or other of the diverticula may lack a cavity (fig. 5, B).

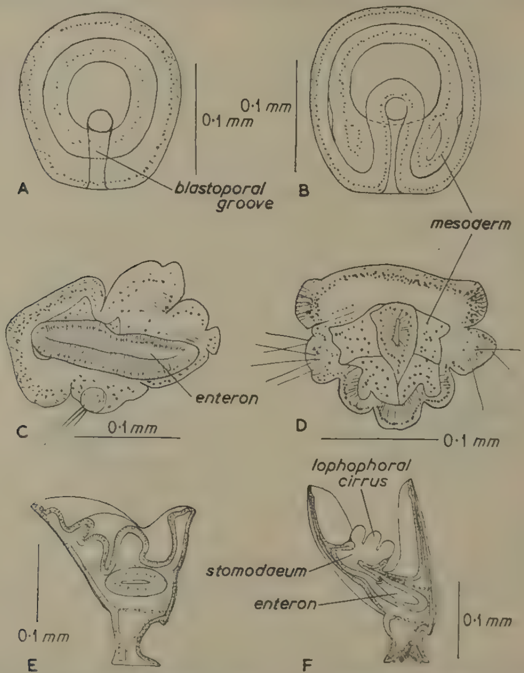


FIG. 5. A, late gastrula with blastoporal groove. B, reconstruction of late gastrula, showing origin of mesodermal masses cut out by the blastoporal groove. C, sagittal section of late embryo, showing a group of setiferous cells and the long enteric mass. D, horizontal section of intermediate larva, showing two pairs of mesodermal masses. E, reconstruction of young adult before the break-through of the stomodaeum; brachial lip rudiment still withdrawn; anterior and posterior invaginations present; a solid section. F, young adult with continuous stomodaeum and enteron; rudiments of cirri; brachial lip in final position; a solid section.

The diverticula quickly separate from the enteric portion and grow forward on each side between the enteron and the ectoderm. The enteron forms into a narrow sac, lying along the length of the body, between the mesodermal bodies that are derived from the enterocoelic sacs. The enteric cavity closes from behind forward, but the extent of closure varies from one specimen to another.

As this rearrangement takes place, the blastoporal groove disappears. The blastopore is a pit, temporarily in contact with the endoderm, and lying near the posterior margin of the ciliated anterior end.

Before the mantle rudiment appears, the mesodermal masses have divided each into an anterior and a posterior portion, the former lying in the presumptive anterior lobe-mantle region, the latter in the presumptive pedicle region. Dorsally and ventrally, the endoderm is in contact with the ectoderm, while laterally the mesoderm separates the two.

When the mantle rudiment appears as a transverse ridge dorsally and dorso-laterally, 4 groups of cells appear in the ectoderm on the posterior border as prospective setiferous cells and serve as striking markers in sections (fig. 5, C).

As the mantle develops, its mesoderm becomes indefinite and rather difficult to distinguish from surrounding ectoderm cells. In the stalk, however, the two hollow mesodermal bodies are distinct for some time, one each side of the well-marked endodermal rod (fig. 5, D).

In time, the stalk mesoderm gives rise to muscles with bands of longitudinal fibres, lying ventro-laterally to the endoderm. At the anterior end of each band, in front of the setiferous cells, there lies on each side in the transverse plane a sheet of muscle fibres extending upward and outward to the ectoderm. The longitudinal muscles become the adjustors. The differentiation of the vertical sheets is uncertain; suffice it to say at present, that the pedicle end of the divaricator is very close to the forward end of the adjustor immediately after mantle reversal.

The endodermal cells are large and take little stain while the endodermal rod is fully extended. Apart from this and the differentiation of the muscle fibres, details of histological structure in the late larva are very indistinct.

Internal changes during metamorphosis. Permanent attachment of the larva is followed by changes in shape accompanied by quiverings and contractions, especially of the anterior lobe, and tiltings one way or another, which may go on for many hours or even for two days. The total length is shortened and the anterior lobe broadens and flattens.

While these movements are taking place, the gut rudiment shortens, being squeezed forward from the pedicle and backward from the apex of the anterior lobe. It becomes re-arranged roughly at a right angle to its former position and lies with its long axis dorso-ventrally. The cells lose their distinctness and take on the same capacity to stain with Heidenhain-eosin as the cells of ectodermal and mesodermal origin. At times it is difficult to distinguish this shortened gut rudiment from the surrounding, relatively undifferentiated material.

The re-orientation of the gut rudiment is complete at about the time when the central area of the apical lobe is retracted. In sagittal section, this lobe is marked off by the deep crescentic clefts noted externally as crescentic slits that join later. The second-formed of these clefts, the morphologically ventral, projects inward toward the brachial end of the rudimentary gut and forms the stomodaeum. The first-formed cleft disappears, while the middle lobe moves to the peripheral position as the brachial lip rudiment (fig. 5, E, F).

As previously mentioned, the apical lobe, being transformed, comes to lie spread on the inner face of the brachial valve. Sections in the longitudinal vertical plane show a combination of anterior lobe and brachial valve, a thickening of the basal mantle region which accommodates the gut rudiment, the presence of muscles in this thickened region, and a distinction between the pedicle valve and the rest of the body.

The pedicle is distinct and directly connected with the pedicle valve.

At this stage of 6 cirri, and before the stomodaeum opens into the mid-gut rudiment, there are certainly 3 pairs of muscle bands present, including the pedicle adjustors. The other visible pairs are one pair anteriorly and laterally to the mid-gut, the adductors or occlusors, and one pair posteriorly to the mid-gut, the divaricators or diductors.

During reversal of the mantle there is no lumen in the gut rudiment, but a minute cavity soon appears and becomes wider while the changes described above are taking place in the anterior lobe. The transverse section of the body is becoming elliptical and the transforming apical lobe is becoming containable by the mantle. About this time, the first shell secretions are visible as smooth, glossy, white surfaces.

Early growth of the young adult. The body tends to become dorso-ventrally flatter, especially on the brachial side (morphologically dorsal), the pedicle side being rounder. These differences in surface affect the form of the calcareous secretion, the protegulum.

The brachial shell is at first transversely elongated, about 3 or more times as long as broad, anteriorly convex, posteriorly concave, and rounded laterally. Touching it postero-laterally are the rather pointed ends of the pedicle shell, which is arched to a semicircle, rather like half of an inverted, truncated cone. With further growth, the marginal contact of the two shells extends, and the body is increasingly contained within the mantle cavity.

When the shell is first secreted, the pedicle is very short and thick, and between it and the shell is a broad, thick region which appears to be formed from that part of the larva where the mantle, stalk, and neck are continuous. With mantle reversal, this part becomes rather swollen and the paired, young-adult muscles lie in it. Mere external observation is insufficient at this moment to show the proper composition of this region. The mantle edge is thick and projects beyond the calcareous plates: the latter meet at their free edges only after much further growth.

Mesodermal cavities. As has been stated, the first mesodermal cavities disappear early and, apart from the muscle-fibres, the mesoderm has, in section, an undifferentiated appearance. Just before the time that the stomodaeum opens into the mid-gut rudiment, spaces appear in the mesoderm between the gut and the muscles, and between the muscles and the ectoderm. The walls of these spaces appear, in places, to have no lining, the muscle-fibres being nakedly exposed to whatever fluid may be in the spaces.

The cavities between the gut and the muscles soon show a lining and finally approach each other to form dorsal and ventral mesenteries suspending the gut. What relation these have to the original enterocoeles is not evident.

The gastric caeca project into the spaces, making their first appearance at about 10 pairs of cirri.

At about 6 pairs of cirri, two simple, thick-walled, ciliated ducts lead, one from each body-cavity or coelom, to the mantle-cavity, lying nearly longitudinally in the angles of the mantle-cavity. They are posterior to the mouth, generally straight, and uniform in diameter.

A single pair of ducts has been found in specimens as large as 1·5 mm long and 1 mm broad. At 2·5 mm long and 2 mm broad, a second shorter pair has been found behind the mouth and near the pedicle valve.

Mesodermal spaces in the cirri have been seen as traces at the stage of 6 cirri. This phase is quite long, the body becoming relatively large and the cirri long before any increase in number of the latter takes place. Meanwhile quite considerable changes take place inside.

At the stage of 4 pairs of cirri, the blood-canals of the cirri are well established, with endothelium and trough-like basement membrane which may be elastic and act as a resister to the contraction of the longitudinal muscle-fibres lying between the trough and the endothelium. The canals are continuous with the peri-oesophageal sinuses, the whole apparatus being much like that described in *Terebratella* (Percival, 1944, p. 19).

By the time 6 pairs of cirri have developed, the gullet has a musculature of longitudinal and circular fibres which can be followed on to the stomach as far as the gastric caeca. Evidently this alimentary musculature is derived from the inner mesodermal epithelia of the body-cavities.

COMPARISON OF THE DEVELOPMENT OF *TEGULORHYNCHIA* WITH THAT OF *TEREBRATELLA*

When comparing the transformation of the apical lobe of *Terebratella* and of *Tegulorhynchia*, it is clear enough that the former process can be regarded as a very much telescoped version of the latter. The rather slow, detailed succession of events seen in *Tegulorhynchia* helps to clarify obscurities in the other, such as the way in which the cirri come to lie on the margin of the mouth, the way in which the brachial lip appears, and the way in which the anterior lobe comes to lie on the inside of the brachial valve of *Terebratella*.

The arrangement of the long setae of the larvae of *Tegulorhynchia* has been shown to be a means of identifying the parts before, during, and after mantle reversal, and by relating these to the adjustor muscles, the erroneous orientation of *Terebratella inconspicua* has been corrected (Percival, 1953).

The development of *Tegulorhynchia* is the more primitive, particularly in the establishment of the young adult. However, it is not yet clear what relation the position of the mouth bears to the site of blastoporal closure.

It is certain that the site of the closed blastopore is last seen in *Terebratella* and *Tegulorhynchia* on the mid-ventral side of the anterior lobe. The stomodaeal invagination appears in *Terebratella* after the anterior lobe has been re-formed on the inner face of the brachial valve, while in *Tegulorhynchia* it occurs on the ventral side of the apex of the apical lobe before the re-formation of the lobe.

While in *Tegulorhynchia* the buccal lip can be followed as an elaboration of the central, apical area of the apical lobe, presumably including that part which carried the apical tuft of cilia, in *Terebratella* the buccal lip appears rather suddenly as an expansion into the oral opening of the anterior border lying between the members of the eighth pair of cirri and before the ninth appear.

The transformation of the apical lobe of *Terebratella* takes place within the more or less completely closed valves, while in *Tegulorhynchia* the process is exposed throughout, the valves closing normally only when the animal is sharply disturbed.

NOTES ON THE SETTLEMENT OF BRACHIOPOD LARVAE

It has been mentioned that the shells of live *Tegulorhynchia* carry varying numbers of the same species and of *Terebratella* and of *Pumilus*, that *Tegulorhynchia* are more commonly found on the undersides of large stones and rarely in a position where *Lithothamnion* grows on them. On the contrary, *Terebratella* is abundant higher on the sides of large stones and under stones nearer the surface of the pool than those carrying the rhynchonellid. It is clear that *Tegulorhynchia* requires conditions for settlement and metamorphosis which are more precise than those needed by *Terebratella*. In the laboratory, this was clearly indicated. *Terebratella* settled more readily on live shells of *Tegulorhynchia;* it also transformed there more readily.

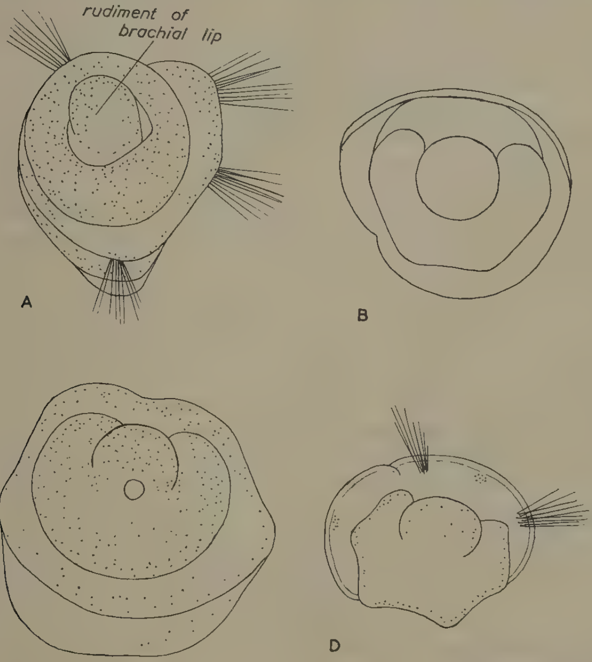
Terebratella readily settled on new surfaces of chipped rock and underwent mantle reversal, while *Tegulorhynchia* did not. The latter settled, however, on old surfaces of the rock chippings and underwent reversal.

Tegulorhynchia would not readily settle on a new glass surface. In 10 days, of several hundred larvae put into fresh sea-water in finger-bowls, about half settled in two bowls and a few score settled in a third. None underwent mantle reversal. The mantle rudiments finally became swollen and transparent, and what were not preserved ultimately died.

Larvae placed in fresh sea-water, in dishes which had held sea-water for about 2 weeks, settled on the glass and underwent partial mantle reversal. The setae were held horizontally by the thickened mantle edge but finally death supervened without further morphological change.

Pathological partial reversal proceeded as far as the outline of 6 cirral rudiments without retraction of the middle of the apical lobe and with the accompaniment of peculiar features internally. The most striking of these internal features was the failure of the shortened enteric rudiment to swing round through a right angle to lie dorso-ventrally, as is normal. Thus, the apical obe formed a large protruding mass surrounded by the expanded mantle udiment. This failure of the gut rudiment to change position affected the ction of the muscle rudiments, which were unable to withdraw the apical nass so that the mantle could continue its reversal and enclose the changing pical mass.

Other interesting accompaniments of incomplete metamorphosis are useful to show the relation between the centre of the apical lobe and the brachial fold. Fig. 6 shows various positions at which change had stopped, although he settlement took place on an old surface where other individuals attached hemselves and completed their metamorphosis. Whether or not that sort f thing occurs in nature has not been determined.



IG. 6. A, arrested development of sessile animal; partial reversal; brachial lip rudiment not vithdrawn; margin not cleft mid-dorsally. B, arrested development; stomodaeal pit; brachial ip rudiment moving outwards into gap of horseshoe. C, D, arrested development towards essile forms; brachial lip rudiment exerted and moved into gap of horse-shoe; rudiments of cirri conspicuous.

Wilson has inquired into the conditions which govern the settlement and netamorphosis of larvae of *Ophelia* and has shown a connexion between acterial population on the substrate and the settlement and metamorhosis: if conditions are suitable settlement occurs and metamorphosis follows. Here is a different state of affairs from what takes place in the brachio pods. Brachiopod larvae swimming in a small amount of water in the laboratory descended from time to time to the substrate, gliding over it apparentl by means of the ventral band of cilia on the mantle rudiment. The anterio lobe was moved about the neck as though testing the ground. Movemen stopped and started and the animals sometimes took to the water agai This might continue for many hours or an animal might suddenly rais itself on the tip of the pedicle and stay indefinitely. The mantle rudimen appeared in these cases to begin reversal, since the setae projected sideway much more than during swimming: this may have been merely an accommodation to the closeness of the substrate. The anterior lobe commonl began to show flattening and pulsations, but even after hours of this kind o behaviour, a slight disturbance with the tip of a fine needle or a jet of wate from a fine pipette would set the animal off swimming; clearly, there ha been no attachment.

Attachment is evident from the accumulation of mucous secretion roun the end of the pedicle. Incomplete reversal is commonly associated with th formation of an obvious calyx of mucus moulded internally through th ciliary action of the anterior lobe and defined externally through the adhesio of small detritus. Complete reversal does not involve this large accumulatio of mucus.

The striking aspect of arrested development of this sort is not that th larvae avoid settlement on an unsuitable substrate, but that settlemen commonly takes place and metamorphosis is incomplete.

There are apparently two main but interrelated stages in the metamorphosis of *Tegulorhynchia*, the first being settlement, partial mantle-reversal, indication of the rudimentary cirri, and rearrangement of the gut rudiment; the second being completion of mantle-reversal with envelopment of the anterior lobe, the readjustment of the central portion of the anterior lobe, and the elongation of the rudimentary cirri. The shortening of the gut rudiment and its rotation appear to be involved in the first stage, leading normally to the apposition of the stomodaeal tip with the primary anterior end of the gut rudiment.

Mrs. F. R. Allison (Frances R. Nurse) compiled the section of the paper entitled 'Comparison of the development of *Tegulorhynchia* with that of *Terebratella*' from Professor Percival's notes.

Parts of Mrs. Allison's contribution have been used in the preparation of the Summary.

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Ester Wax 1960: a Histological Embedding Medium

By H. F. STEEDMAN

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SUMMARY

1. A modification of the original formula (Steedman, 1947) is given which still retains the softness of infiltrated tissues.
2. The section, both wax and specimen, flattens on water.
3. The sections of a ribbon adhere during flattening.
4. Adhesion of sections to the slide is good.
5. The room temperature for successful ribboning may vary from 17° to 25° C or higher.

THE softness of infiltrated tissues when diethylene glycol distearate is the infiltration medium has been the subject of favourable comment by Orton and Post (1932), Cutler (1935), Steedman (1947), Chesterman and Leach (1956), and others. The considerable hardness of this material compared with paraffin wax and its slightly lower melting-point (48° C) constitute additional attractive features which have induced research workers to give it most thorough examination. Alone, diethylene glycol distearate has weaknesses which preclude its use as an infiltration medium. The 1947 (Steedman) formula produced a medium of excellent ribboning properties, but gave poor flattening of sections, and showed separation of the sections of a ribbon during flattening. The modification by Chesterman and Leach (1956) showed improvement of flattening, though this was induced by the use of a detergent (teepol). The use of a detergent generally leads to detachment of sections during staining, and this had to be counteracted by running slides through a weak bath of celloidin. The sections also separated from each other during flattening as they did when the earlier formula was used.

Variation in the properties of manufactured stearates has caused some difficulty in producing a stable infiltration medium, and though the products today are much better than they were in 1947 and 1956, there is still some variation. A pure stearate produced as a commercial product would be so expensive that its use as an infiltration medium would be unattractive. Manufacturers experience difficulty in obtaining a sufficiently pure stearic acid. The amount of palmitic and oleic acids present in commercial samples of stearic acid may vary considerably, and as a result the esters made from these samples vary too. In spite of this fluctuation, known and expected, the ester wax modification below shows a physical stability which has led to consistent results.

Ester wax 1960

diethylene glycol distearate	60 g
glyceryl monostearate	30 g
300 polyethylene glycol distearate	10 g

[Quarterly Journal of Microscopical Science, Vol. 101, part 4, pp. 459–62, 1960.]

The introduction of glyceryl monostearate to the formula changes the 1947 mixture from its amorphous and translucent condition to one which is crystalline but opaque. The 300 polyethylene glycol distearatc imparts good section-to-section adherence, and excellent flattening. The mixture is based upon esters with the following characters:

Diethylene glycol distearate. The type used in the 1960 formula is a very hard, white, translucent to transparent material. It breaks with a conchoidal fracture, and is sold as digol distearate, in small, broken lumps. (British Drug Houses, Poole, Dorset, England.)

Glyceryl monostearate. Purchased as a white powder. 300 polyethylene glycol distearate. A soft, yellow material, greasy to the touch; a little stiffer than lard. (Watford Chemical Co., 22 Copperfield Road, Canal Road, London, E. 3.)

To make the wax, first melt the diethylene glycol distearate and heat it until it is clear. Then add the glyceryl monostearate, and when that is dissolved add the 300 polyethylene glycol distearate. Filter through a Barcham Green 904 filter paper. It is an advantage to hold the paper in a ring rather than in a filter funnel, because this permits better and faster filtration. The wax may be obtained made up and ready to use from the British Drug Houses, Poole, Dorset, England.

The following details apply to the mixture:

Ester wax 1960

Melting-point, 48° C.

Sections, flat, may be obtained at from 1 to 12 μ at a room temperature of 17° to 27° C.

Ribbons may be obtained at from 1 to 10 μ at a room temperature of 17° to 27° C.

Static electricity. The wax shows no charge.

Solubility. The wax is soluble in 95% ethanol, aqueous; ethanol; *n*-butanol; 2-ethoxy-ethanol (ethylene glycol monoethyl ether); monochlorisothymol; acetone; cedarwood oil; xylene; chloroform; and in the majority of esters, ethers, ketones, alcohols, hydrocarbons, and chlorinated hydrocarbons.

Clearing agents. The following are recommended: 2-ethoxy-ethanol, *n*-butanol, ethanol, monochlorisothymol, and xylene.

Block hardening time. A block measuring 20×10×10 mm is ready for cutting into 10 μ sections in about 1 h after pouring at room temperature 20° C.

Cutting speed, 30 to 50 sections a minute.

Viscosity, approximately 28 to 30 centistokes at 55° C.

Appearance, a white to cream, opaque wax.

Knife bevel angle. This ranges from 20° to 30°.

Section-to-section adhesion, good, but with a slight tendency to separate during flattening.

Section flattening, very good.

Section adhesion to glass, good.

Keeping properties when molten. A tendency to flocculate after 7 to 10 days at 48° C may be removed by heating to about 80° C.

Infiltration. After an appropriate length of time in the clearing agent, the specimen should be transferred to a bath of clearing agent with the wax dissolved in it. A mixture of equal parts of clearing agent and molten wax may be sufficient for most specimens, but should a more gradual change from the clearing agent to pure molten wax be required, the mixtures used might be 75 volumes of clearing agent to 25 of wax; then 50:50, then 25:75, and finally pure wax. Even finer gradations may be used should they be considered necessary.

The time of infiltration depends on the size of the specimen, but a slice of liver about 4 to 5 mm in thickness will take about 4 h without any stirring. The time would be reduced by 25 to 30% should stirring be employed. Specimens may be kept overnight in the molten wax without damage.

Block making. Adjust cold L-pieces to an appropriate size and place on a piece of glass or metal. Heat some solid ester wax as received from the supplier until it is melted at about 10° C above its melting-point. Pour the molten wax into the L-pieces. When a thin, opaque film is seen on the inside of the metal, add the infiltrated specimen, and adjust as required. It is an advantage to place the specimen eccentrically rather than in the middle of the block.

As the block cools, keep the surface liquid by touching it with a heated spatula or by quickly turning a lighted Bunsen burner on to it for a fraction of a second. The block may be cooled on the bench, or it may be placed in a shallow dish with ice cubes.

A parting agent such as glycerol is not needed if the L-pieces are clean.

Block trimming. Ester wax 1960 is a brittle or crumbly compound when cut n thick slices; therefore only thin slices should be taken off during trimming. A single-edge razor-blade is best for trimming.

Attachment of block to block-holder. A flat, heated spatula should be used, as with paraffin wax.

Cutting. A knife with a bevel angle of 25° is suitable. Above 30° the wax begins to show excessive compression. The cutting speed for ribboning at a room temperature of 20° C (68° F) is between 30 and 50 sections a minute.

Flattening sections. Smear a slide with albumen, or take a perfectly grease-free slide and do not use any albumen at all. Run a film of water on to the slide. On the film place the ribbon or section. Place the slide on a hot plate at 45° to 50° C, or hold it on the surface of a bowl of hot water at the same temperature. The wax will soften and flatten in about 20 sec; the specimen will flatten in about 40 to 60 sec. Drain the slide of excess water, and dry it at about 40° to 45° C for an hour or two, or overnight. During flattening the section will expand and will reach and slightly surpass the measurement of the block face.

Adhesion of sections. Very clean, grease-free slides may be quite suitable without the addition of any adhesive. Tissues vary in their capacity for adhesion to glass and some, such as brain, chitin of arthropods, cuticle of

Ascaris, and oviduct of Amphibia, may show a strong tendency to come awa from the slide during staining operations. In these circumstances Mayer' albumen, Baker and Jordan's (1953) adhesive albumen, or amylo-pecti (Steedman, 1957) may be used with advantage.

Staining of sections. Remove the wax from dried sections with xylene, an continue as with paraffin wax.

Histological effects. The appearance of tissues infiltrated with ester wax 196 is comparable with, and in many respects superior to, that of tissues infiltrate with paraffin wax. This is attributable to the chemical composition and als to the physical structure. The hardness of ester wax reduces considerabl the intercellular and inter-tissue movements of material at the moment o cutting. As a result a smoother appearance is imparted to the finished, stained mounted section.

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Tropical Ester Wax 1960

By H. F. STEEDMAN

(*From the Department of Zoology, The University, Glasgow*)

SUMMARY

1. A modification of the standard ester wax 1960 is given which enables sections to be cut at a room temperature of 17° to 37° C (62·6° to 98·6° F).

2. The melting-point of tropical ester wax is 50° C.

3. Ribboning and flattening of sections is good, from 1 to 10 μ.

MICROTOMY in a hot room, whether in the tropics or in an overheated building, is generally accomplished by the use of infiltration waxes with high melting-points. This means that during infiltration the specimen is subjected to temperatures about 56° to 60° C. High infiltration temperatures lead to shrinkage and excessive hardness of the specimen, and for these reasons it is an advantage to keep infiltration temperatures as low as possible.

When a solid wax is subjected to increasing temperatures it reaches a point at which it softens and will form a liquid drop. The lowest temperature at which this takes place is the melting-point. When a quantity of the same wax in the liquid condition is gently cooled it reaches a point at which it becomes cloudy, and then it sets or becomes solid. This is the setting-point. With any given wax there is generally a difference of a few degrees between the melting-point and the setting-point. The setting-point is lower. It is thus possible, by prolonging the setting period, to keep a wax liquid below its melting-point, though such a condition cannot be maintained indefinitely.

This principle is employed in tropical ester wax 1960, which has the following formula:

Tropical ester wax 1960

diethylene glycol distearate	60 g
glyceryl monostearate	30 g
triethylene glycol monostearate	10 g

The mixture will keep liquid at 50° C for about 4 days. After that it begins to flocculate. The liquid wax will become more cloudy if kept at that temperature, but it may be heated to 80° C and filtered. The filtrate will keep clear for another 4 days or more, after which it should be heated and refiltered. It is possible to infiltrate a specimen for any time up to 4 days or even longer, at 50° C.

Sections and ribbons may be cut at 3 to 10 μ when the room temperature is as high as 37·5° C (99° F), after the block has been at that temperature for a week. By keeping the block and knife in cool conditions, such as in a refrigerator at about 15° C, good sections and ribbons may be obtained in a room with a temperature even higher than 37° C.

[Quarterly Journal of Microscopical Science, Vol. 101, part 4, pp. 463–4, 1960.]

The sections and ribbons cut in a hot room should always be placed on a soft, rough-surfaced paper, such as duplicating paper. If placed on a smooth, highly calendered paper, the sections will show a much greater tendency to adhere to the paper or to melt.

Tropical ester wax 1960 may be obtained made up and ready for use from the British Drug Houses, Poole, Dorset, England.

Apart from the capacity to withstand higher room temperatures, the wax is similar in all respects to the standard ester wax 1960 (Steedman, 1960).

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On the Interpretation of Phase-contrast Images

By W. H. STEEL *and* Y. T. TCHAN

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SUMMARY

It is shown that the simple theory of phase-contrast microscopy may be extended to take some account of the size of the object by the introduction of an 'overlap factor' which expresses the fraction of the diffracted light that passes through the phase-changing region of the phase plate. The extended theory can explain observed effects that cannot be explained even qualitatively if overlap is ignored. The overlap factor is given for disk-shaped objects and an annular phase plate.

INTRODUCTION

THE full mathematical treatment of phase contrast is fairly complex and microscopists usually rely on a simplified theory. This considers first a point source of light, and the complex amplitude imposed by the object is followed through the optical system; it is then assumed that an extended source gives a similar result. The treatment is given either in complex algebra or as the Argand or 'vector' diagram that represents these complex numbers (Barer, 1952). Many observed results can be explained in this way, but there are obvious limitations, as no account is taken of the size or shape of the object. Thus no explanation is given of the haloes surrounding sharp edges or of the loss of contrast towards the centre of the image when the object is not very small.

Hopkins (1953*a*) has shown that the assumptions on which the simple theory is based reduce to:

(1) all the direct light, and none of the diffracted light, passes through the phase-changing region of the phase plate;

(2) all the diffracted light falls within the aperture of the image-forming system.

The second assumption ignores the limited aperture of the objective and therefore implies that the objective has unlimited resolving power, so that, if no phase system were present, the image would be an exact reproduction of the object. The effects of a limited aperture are similar in both the phase microscope and the ordinary microscope and will not be considered here.

The first assumption has no equivalent in the theory of ordinary microscopes. In many practical cases it is not even approximately true, as shown by the inability to explain the phenomena mentioned above. To explain these results a theory such as that given by Ramsay (1952) is required. He considers the case of an extended object, an optical system with a limited aperture, and a circular phase plate, and his results then agree with observation. However, his theory is too complex for practical use by microscopists.

[Quarterly Journal of Microscopical Science, Vol. 101, part 4, pp. 465–73, 1960.]

In this paper we develop a simpler theory that follows the form of the elementary theory, well known to microscopists, and show that this can explain the loss of contrast at the centre of large objects. Such objects diffract an appreciable amount of light through small angles and, as both the source (condenser diaphragm) and the phase-changing region have a finite size, there can be a considerable overlap of diffracted images of the source on to the phase-changing region. This 'overlap factor' can be introduced either into the algebra of the simple amplitude theory or into Barer's Argand diagrams.

Experimental Results

Although the simple theory suggests that the image contrast obtained is independent of the size of the object, it is well known that objects having the same phase advance or retardation but different sizes give rise to different image contrasts. When the object is also absorbing, the image of a larger object may even show a reversed contrast as compared with that of a smaller one.

A simple experiment can be used to demonstrate these effects. An air-dried smear of an unidentified amoeba mixed with bacteria is stained red with erythrosin and, after drying, mounted in Reichert immersion oil (refractive index at D line, 1·515). This preparation provides objects of two distinct sizes, both having refractive indices lower than that of the mounting medium and hence giving small phase advances. The coloration enables the absorption in the objects to be varied by the use of light of different colours; their transmission is almost unity in red light but much lower in green. Although the use of light of different wavelengths gives slightly different properties to the phase system (a quarter wavelength retardation in green light becomes just over a fifth of a wavelength in red), this should make only a negligible difference to the results obtained.

The preparation is now examined with a phase system that advances and absorbs the direct light; such a system is known as *positive phase contrast*. The images in red light of both the amoebae and the bacteria appear brighter than the background, as would be expected; we shall call this *positive image contrast*. In green light, however, although the bacteria still appear with positive but reduced contrast, the larger amoebae appear in negative contrast (darker than the background).

By the simple theory, the images should always have the same contrast if both specimens have approximately the same phase change and absorption. Even if these are not quite the same, no object that gives a small advance of phase should give an image with negative contrast with a positive phase contrast system. Fig. 1 shows the calculated image contrast C' as a function of the amplitude transmission a of the object for different phase advances ϕ of the object; C' is defined as $(B'_S-B'_F)/B'_F$, where B'_S and B'_F are the illuminations in the image plane at the image of the specimen and across the background. The results are given for positive phase objectives having amplitude transmissions for the direct light of $k = 1$ or $\frac{1}{2}$ and a phase advance of $\frac{1}{2}\pi$. In no case are negative contrasts predicted.

The same result can be seen on Barer's 'vector circle' (1952) as shown in fig. 2. For a phase system giving a $\frac{1}{2}\pi$ phase advance to the direct light, the reference origin O' for the image lies between M and P, so that $O'M/PM = k$. The complex transmission of the object is represented by OF of length a and angle ϕ; ϕ is known to be small. It is obvious that, for ϕ positive, the amplitude in the image, represented by the length $O'F$, is always greater than the background amplitude $O'M$ (unless ϕ is greater than $\frac{3}{2}\pi$, which is not true in the experiment above).

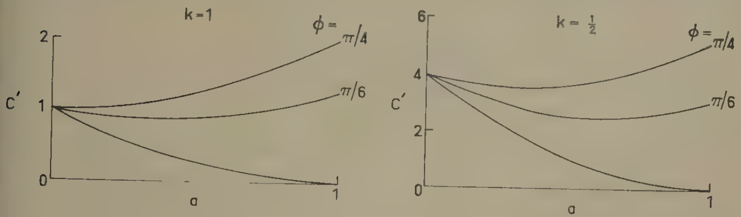

FIG. 1. Image contrast C', derived from elementary theory, as a function of object amplitude transmission a and phase ϕ, for positive phase contrast.

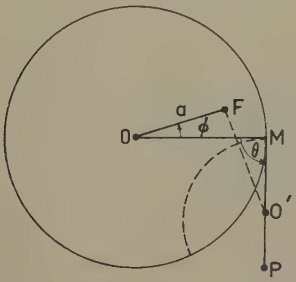

FIG. 2. Argand diagram for positive phase contrast and an object giving a small phase advance ϕ.

The failure of the simple theory to explain such observed results suggests that at least one of the assumptions of this theory is not satisfied. A clue to a better explanation is given by studying the theoretical image given by a microscope with no phase change but only absorption in the phase region. In this case negative contrasts are predicted, as shown in fig. 3. If a theory which assumes no phase contrast gives a better prediction of the image of fairly large objects (such as the amoebae) obtained with a phase microscope than does a phase-contrast theory, it is apparent that, in such cases, so much of the diffracted light from these objects passes through the phase-changing region, along with the direct light, that very little effective phase difference is introduced between the two beams. The first assumption of the simple phase-contrast theory therefore needs modification.

EXTENDED THEORY

The discussion above suggests that the correct result is given by a theory intermediate between the usual phase-contrast theory and that of the ordinary microscope. To obtain this theory an 'overlap factor' ϵ is introduced to represent the fraction of the diffracted amplitude that passes through the phase-changing region.

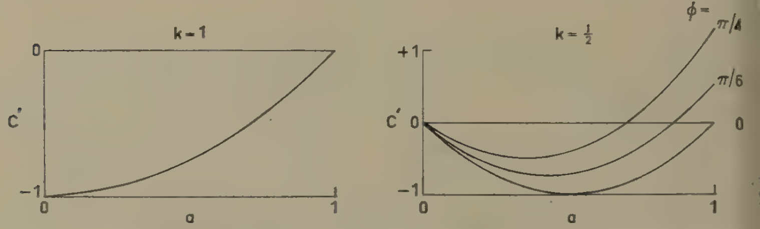

FIG. 3. Image contrast C' as a function of object transmission a when no phase change is introduced by the phase system.

The object is assumed to consist of a small region of amplitude transmission a ($a \leqslant 1$) and phase advance ϕ on a background of unit amplitude. The complex amplitude leaving the object plane can be broken up into an amount 1 from the background, which gives the direct light, and an amount $ae^{i\phi}-1$ giving the diffracted light.

All the direct light and a fraction ϵ of the diffracted light passes through the phase-changing region whose complex transmission is $ke^{i\theta}$. The remaining fraction $1-\epsilon$ of the diffracted light passes through the phase plate with unit transmission. The complex amplitude at the image is then

$$A' = ke^{i\theta}\{1+\epsilon(ae^{i\phi}-1)\}+(1-\epsilon)(ae^{i\phi}-1).$$

The illumination at the image is given by $|A'|^2$ or

$$B' = (1-\epsilon)^2(1+a^2+k^2)+\epsilon^2k^2a^2+2(1-\epsilon)(\epsilon-1+a^2)k\cos\theta+$$
$$+2(1-\epsilon)\{\epsilon k^2-1+\epsilon+(1-2\epsilon)k\cos\theta\}a\cos\phi+2(1-\epsilon)ak\sin\phi\sin\theta.$$

The illumination across the background is k^2, so the image contrast is

$$C' = B'/k^2-1.$$

These expressions have been used to calculate the image contrasts shown in fig. 4 which are based on $\epsilon=\frac{1}{2}$, $\theta=\frac{1}{2}\pi$, and $k=1$ and $\frac{1}{2}$. It is seen that, with this amount of overlap, negative image contrasts may be predicted for objects with a small phase advance and some absorption.

MODIFIED ARGAND DIAGRAM

Just as the algebraic theory may be extended to include overlap, a similar extension may be represented on Barer's 'vector diagram'. The steps used are shown in fig. 5. As usual, the unit circle of centre O represents the domain of the object complex amplitude $ae^{i\phi}$ with OM representing the direction of zero phase. The reference centre for image amplitudes is O', lying a distance k from M along a line MP which makes an angle $OMP = \theta$ with OM, θ being the phase advance of the phase plate, here shown as $\frac{1}{2}\pi$.

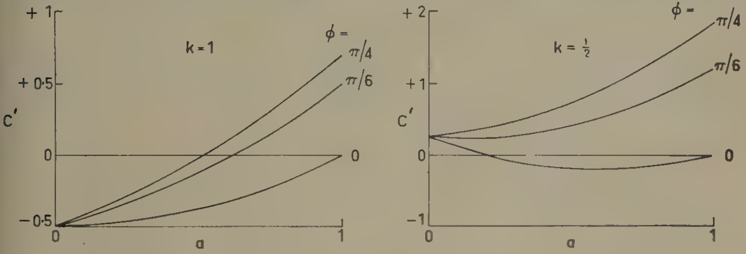

FIG. 4. Image contrasts as in fig. 1 but with allowance for an overlap $\epsilon = \frac{1}{2}$.

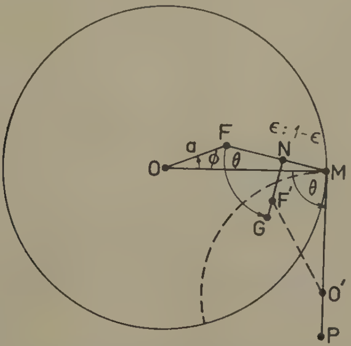

FIG. 5. Argand diagram for positive phase contrast, modified to include overlap.

The diffracted light from the object is represented by MF. This is divided in the ratio ϵ to $1-\epsilon$ by the point N. The fraction $1-\epsilon$, represented by MN, passes through a region with no phase-changing material and is transmitted unchanged, but the remaining portion NF passes through the phase-changing and absorbing region and is rotated in phase by the same angle θ to NG and reduced in length by the factor k to give finally NF'. The image complex amplitude finally obtained is represented by the vector sum $O'M+MN+NF'$ and has an amplitude given by the length $O'F'$.

It is seen that this amplitude can be less than the amplitude $O'M$ of the background and that negative contrasts can be obtained. Further, a combination of values of object phase ϕ and phase-region transmission k that gives negative contrasts for small values of a, the object transmission, will give positive contrasts for a large. This explains why the image of the amoebae has positive contrast in red light (a near unity) but negative contrast in green light (a small).

OVERLAP FACTOR

The value of the overlap factor ϵ that should be used to interpret any particular observation depends on the size and shape of the object and of

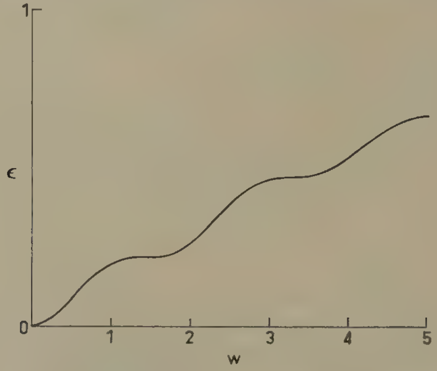

FIG. 6. The overlap factor ϵ for annular source and phase system for circular disk objects of diameter w.

the phase system (condenser diaphragm and phase-changing region). Phase systems are usually annular, with their inside and outside diameters in the ratio 3 to 4 approximately.

It is impossible to give values of ϵ for every irregularly shaped object that may occur in practice. But, as a rough approximation, the values for circular disk objects of the same area as the natural object may be used. This is reasonable with annular phase systems, since these average the overlap of the diffracted light for all orientations and thus should not be greatly affected by irregular shapes of the object.

Fig. 6 gives the overlap factor ϵ for uniform circular disk objects in terms of their diameter. These are calculated from the theory given in the appendix for a source and a phase region, both of which have their inner diameter three-quarters of their outer diameter. The results also depend on the value of b shown in fig. 7; this is the ratio of the objective aperture at the phase plate to the phase-ring aperture. The diameter of the disk object is given by a value w which depends on b and also relates the object size to the limit of resolution of the objective, given by $0{\cdot}61\lambda/\text{N.A.}$ If the disk has a diameter

n times this limit of resolution, the value of w to be used in fig. 6 is given by $w = n/b$.

CONCLUSIONS

It has been shown that the usual elementary theory of phase contrast in the form familiar to microscopists can be extended rather simply to take account of the size of the object by the introduction of an overlap factor which expresses the fraction of the diffracted light from the object that passes through the phase-changing region. This factor is given for circular disk objects for a phase annulus of particular proportions, but, as the whole theory is a very rough approximation, the results could be used for most normal phase objectives and any objects of the same area but different shapes.

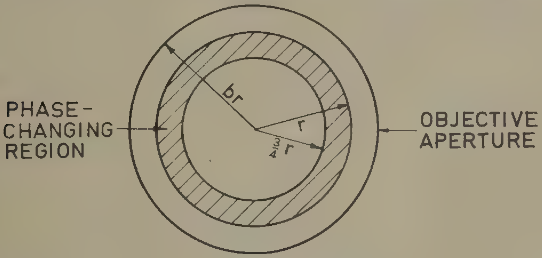

FIG. 7. Relative apertures of the objective and phase annulus, the objective aperture being the circle formed by the cone of rays from the edge of the objective in the plane of the phase plate.

To derive the overlap factor, a more complete treatment is given in the appendix. At the same time an indication is given of the size of the errors involved in the simple overlap theory. Although these are not negligible, they are much smaller than the errors of the usual simple theory.

APPENDIX

To derive the overlap factor ϵ, a fuller treatment of the theory of image formation is required. The notation used is that of Steel (1959); points at the source are represented by position vectors $\mathbf{u}$ and $\mathbf{v}$, in the object plane by $\mathbf{x}$, $\mathbf{y}$, at the pupil or phase plate by $\mathbf{u}'$, $\mathbf{v}'$, and in the image plane by $\mathbf{x}'$. All of these are 'reduced vectors' as defined in that paper.

In this notation, the illumination at $\mathbf{x}'$ in the image plane is given by Hopkins (1953*b*) as

$$B(\mathbf{x}') = \frac{1}{8\pi^3} \iiint_{-\infty}^{+\infty} g'(\mathbf{v}) l(\mathbf{u}') l^*(\mathbf{v}') f(\mathbf{u}'+\mathbf{v}') f^*(\mathbf{v}'+\mathbf{v}) e^{i(\mathbf{u}'-\mathbf{v}')\cdot\mathbf{x}'}\, d\mathbf{v}\, d\mathbf{u}'\, d\mathbf{v}',$$

where:

(1) $g'(\mathbf{v})$ is the brightness distribution across the effective source;

(2) $L(\mathbf{x})$ is the complex amplitude transmission in the object plane, $l(\mathbf{u}')$ its two-dimensional Fourier transform, and $l^*(\mathbf{u}')$ the complex conjugate; and

(3) $f(\mathbf{u}')$ is the complex amplitude transmission of the image-forming system, including the phase plate.

For this expression to give the same result as the simple theory, $B(\mathbf{x}')$ should be expressible as a squared modulus such as, to a constant factor,

$$\left|\frac{1}{4\pi^2}\iint\limits_{-\infty}^{+\infty} g'(\mathbf{v})l(\mathbf{u}')f(\mathbf{u}'+\mathbf{v})e^{i\mathbf{u}'\cdot\mathbf{x}'}\,d\mathbf{v}d\mathbf{u}'\right|^2.$$

This is not true in general, but, if certain assumptions are made as to the form of the object, source, and phase system, an approximate agreement can be found.

We make these assumptions:

(1) The source is of uniform brightness over a region $p(\mathbf{v})$ and, when imaged at the phase plate, it covers the same area as the phase-changing region, which is then $p(\mathbf{u}')$. Thus

$$g'(\mathbf{v}) = bp(\mathbf{v}),$$

where b is a constant and $p(\mathbf{v})$ is unity over the source and zero for other values of $\mathbf{v}$. Obviously $p^2(\mathbf{v}) = p(\mathbf{v})$.

(2) The object consists of a small region $S(\mathbf{x})$ of uniform complex transmission $ae^{i\phi} = \alpha+1$, where $S(\mathbf{x}) = 1$ over the object and zero elsewhere. This object lies in a uniform field of unit transmission. Then

$$L(\mathbf{x}) = 1+\alpha S(\mathbf{x}),$$
$$l(\mathbf{u}') = 2\pi\delta(\mathbf{u}')+\alpha s(\mathbf{u}'),$$

where $\delta(\mathbf{u}')$ is a two-dimensional Dirac delta function and $s(\mathbf{u}')$ is the Fourier transform of $S(\mathbf{x})$.

(3) The pupil transmission is $ke^{i\theta} = \kappa+1$ over $p(\mathbf{u}')$ and unity elsewhere, the pupil being taken as unlimited.

If these values are inserted in the general equation, the result can be expressed in terms of the following integrals:

(i) $\displaystyle\frac{1}{2\pi}\int\limits_{-\infty}^{+\infty} p(\mathbf{v})\,d\mathbf{v} = G$, the reduced area of the source or phase-changing region;

(ii) $$\frac{1}{4\pi^2}\iint\limits_{-\infty}^{+\infty} p(\mathbf{v})p(\mathbf{u}'+\mathbf{v})s(\mathbf{u}')\mathrm{e}^{i\mathbf{u}'\cdot\mathbf{x}'}\,d\mathbf{v}d\mathbf{v}' = \frac{1}{2\pi}\int\limits_{-\infty}^{+\infty} P(\mathbf{x})P(-\mathbf{x})S(\mathbf{x}'-\mathbf{x})\,d\mathbf{x}$$
$$= GH(\mathbf{x}');$$

(iii) $$\frac{1}{8\pi^3}\iiint\limits_{\infty}^{+\infty} p(\mathbf{v})p(\mathbf{u}'+\mathbf{v})p(\mathbf{v}'+\mathbf{v})s(\mathbf{u}')s(\mathbf{v}')e^{i(\mathbf{u}'-\mathbf{v}')\cdot\mathbf{x}'}\,d\mathbf{v}d\mathbf{u}'d\mathbf{v}' = GW(\mathbf{x}').$$

Then $\quad B(\mathbf{x}') = bG[|1+\alpha S(\mathbf{x}')+\kappa+\alpha H(\mathbf{x}')|^2+\alpha\alpha^*\kappa\kappa^*\{W(\mathbf{x}')-H^2(\mathbf{x}')\}].$

If this result is compared with that given by the simple theory, the first term has the required form of a squared modulus and agrees with the earlier result if $\epsilon = H(\mathbf{x}')$. It was this result that was calculated for fig. 6 for the centre of the image ($\mathbf{x}' = 0$). However, for the simple theory to be even approximately true, the extra term containing $W-H^2$ should be small. It seems reasonable that this should be so, for the integrals G, H, and W should decrease in value in this order as the regions of integration become smaller.

It is difficult to evaluate $W-H^2$ for annular phase plates, but it has been computed for circular phase and a circular disk object whose diameter is again expressed in terms of w. The results are given in fig. 8. It can be seen that, although the error is not negligible when compared with unity, it is appreciable for only a small range of object sizes.

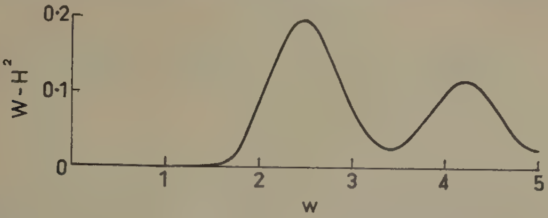

FIG. 8. The factor $W-H^2$, explained in the appendix, from which the error of the elementary theory can be derived.

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The Interstitial Cell in the Testis of the Foetal Sheep

By A. H. BAILLIE

(*From the Department of Anatomy, University of Glasgow*)

With one plate (fig. 1)

SUMMARY

The testes of 18 foetal sheep, of crown–rump length from 2·8 to 40 cm, have been studied by the PAS, Sudan black, and plasmal tests. Typical interstitial cells differentiate from mesenchymal precursors; the nucleus alters first, becoming ovoid and vesicular; then the cytoplasm increases in amount, its processes become fewer and smaller, sudanophil lipid droplets appear, and the result is a lipid-laden epithelioid cell. Two atypical forms of interstitial cell have been noted: the first has groups of eosinophil granules in its cytoplasm, the second is shrunken and has a pycnotic nucleus. The interstitial cell of foetal sheep, unlike that of poikilotherms, contains no glycogen. The PAS-positive polysaccharide / protein granules found in the interstitial cells of adult homiotherms are absent from the interstitial cells of this foetal homiotherm. While sudanophil lipids appear at an early stage, Schiff-positive lipids (plasmalogens, acetal phosphatides, and possibly steroids) are entirely absent from the interstitial cells of the foetal sheep.

INTRODUCTION

UNTIL comparatively recently there has been considerable controversy regarding the precise foetal origin of the testicular interstitium; embryonic sources suggested by various writers for the interstitial cell include leucocytes, lymphocytes, plasma cells, capillary endothelial cells, the sex cords, Sertoli cells, and the coelomic epithelium (Gillman, 1948). Now, however, it is widely accepted, in accordance with Gillman (1948), that the interstitial cell develops from small spindle-shaped elements lying between the seminiferous tubules. This intertubular tissue is derived in the first instance from the general mesenchyme of the primordial testis, but it also receives a contribution of mesonephric mesenchyme accompanying the blood-vessels. It thus appears that the interstitial cell arises from a stellate precursor which is morphologically indistinguishable from the cells giving rise to the cellular elements of ordinary connective tissue.

Although much time and attention have been devoted to the histogenesis of the foetal interstitial cell, there is little information available at present regarding its histochemical characteristics. This work is a study of some of the histochemical features of the foetal interstitial cell; it also seeks to furnish histochemical evidence in support of a mesenchymal origin of the interstitial cell.

MATERIAL AND METHODS

In all, 18 male sheep embryos were studied; the crown–rump length varied from 2·8 to 40 cm. The details are tabulated as follows:

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Crown-rump length (cm)	*No. of embryos*	*Position of testes*
2·8	1	medial to kidney
8·1	1	abdominal
9·7	3	,,
17·5	3	inguinal canal
21·3	2	,,
24·3	1	,,
31·0	2	scrotal
33·0	2	,,
36·0	1	,,
40·0	2	,,

One testis from each animal was fixed intact in a mixture of 90 ml water, 10 ml formalin, and 5 g mercuric chloride, dehydrated in cellosolve, embedded in ester wax, and sectioned at 5 μ. The stains used were haematoxylin and eosin, and also the McManus/Hotchkiss periodic acid / Schiff (PAS) technique (Carleton and Drury, 1957). It has been shown (Vallance-Owen, 1948) that glycogen is as well fixed by formaldehyde fixatives as by alcohol or alcohol/ picric mixtures; the former were found to give vastly superior histological results in the testis, shrinkage of interstitial tissue being minimal by this method.

With the exception of the smallest embryo, the other testis was in each case fixed in formaldehyde-calcium solution (90 ml water, 10 ml formalin, 1 g anhydrous calcium chloride), embedded in gelatin, and sectioned at 10 μ on the freezing microtome. Some frozen sections were coloured in Sudan black to demonstrate total lipids, while others were subjected to Hayes's modification of Feulgen and to Voit's plasmal reaction (Lillie, 1954) to show acetal phosphatides and possibly steroids (Dempsey, 1948).

RESULTS

To avoid repetition, descriptions of the specimens stained with haematoxylin and eosin are presented conjointly with descriptions of the corresponding PAS slides.

Results with haematoxylin and eosin and with PAS. The gonad of the 2·8-cm embryo is a mesenchymal mass, covered with cuboidal coelomic epithelium, located in the dorso-medial angle of the coelom: it is coextensive with the developing kidney. The primordium consists solely of stellate mesenchymal cells (fig. 1, A), whose membranes are well defined by the PAS procedure. Occasional red, refractile, PAS-positive, diastase-resistant granules may be seen in some cells. The nuclei are usually large and oval. A few large hexagonal cells with vesicular nuclei are visible at the periphery of the primordium; these may be the primitive sex cells. No PAS-positive ground substance is visible.

The gonads of the 8·1-cm and 9.7-cm embryos resemble one another closely. The spaces between the differentiating seminiferous tubules are populated with stellate mesenchymal cells resembling those described in the

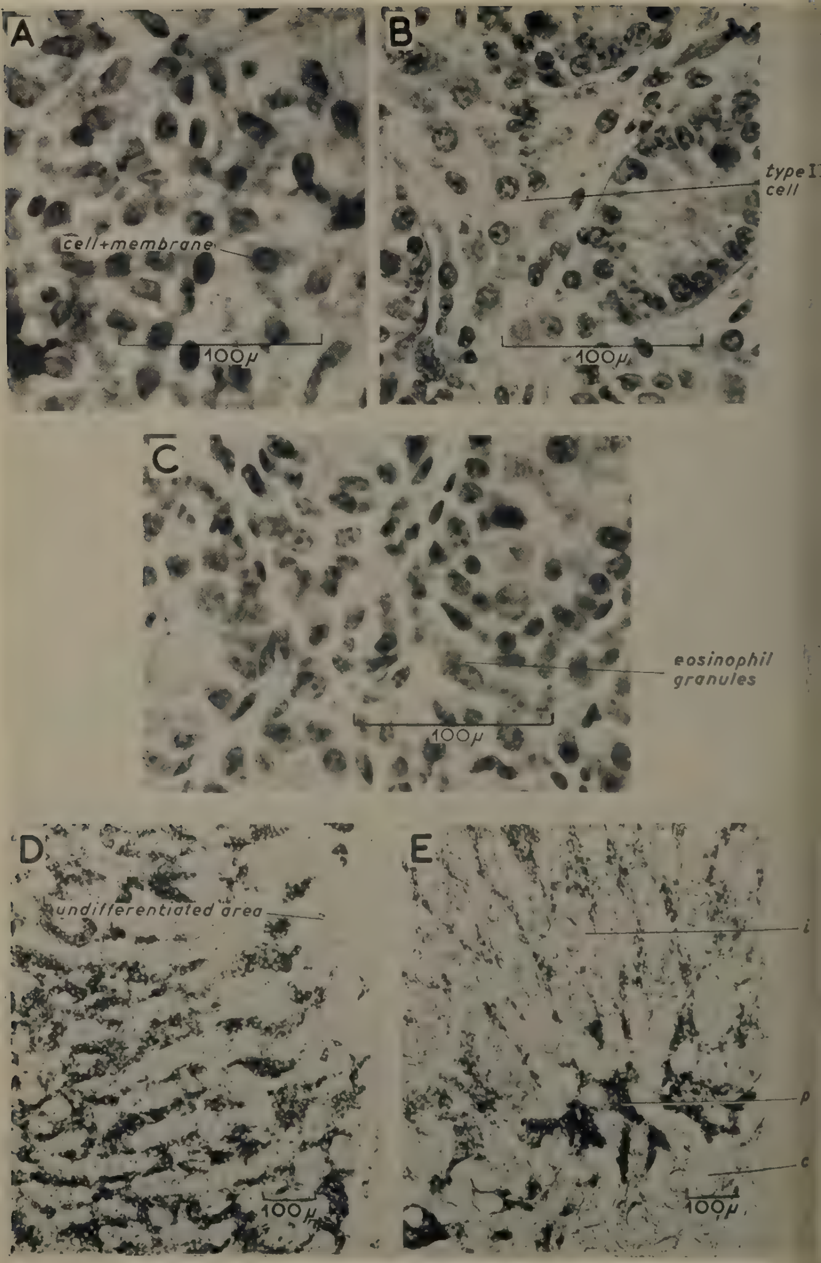

FIG. 1

A. H. BAILLIE

2·8-cm embryo; these cells are connected by transition forms with peritubular fibroblasts on the one hand and with interstitial cells on the other. The latter have abundant hexagonal or rounded cytoplasm, which stains pale pink with eosin; there are no PAS-positive cytoplasmic inclusions. Their nuclei are ovoid and vesicular and possess a characteristic chromatin arrangement.

The first visible event in the differentiation of the typical interstitial cell from its mesenchymal precursor is alteration of the nucleus. The nucleus, originally oval and containing dust-like chromatin, becomes vesicular and round, while the chromatin becomes localized just inside the nuclear membrane: one or more nucleoli become visible. Cytoplasmic differentiation is a slower process than nuclear differentiation. Initially the cytoplasm increases in amount, its processes diminish in size and number before finally disappearing, and the cell-body assumes a polygonal or epithelioid appearance. At a variable stage during this phase (see below), sudanophil droplets make their appearance.

The testes of the 17·5-cm, 21·3-cm, and 24-cm embryos present a uniform histological appearance. There are three recognizable types of interstitial cell:

(1) the most common form has abundant cytoplasm which colours pink with eosin; the cell-membrane stains clearly with PAS (fig. 1, B); the nucleus is vesicular and ovoid.

(2) the second form differs from the first in that it has variable numbers of prominent eosinophil granules in its cytoplasm (fig. 1, C). The granules occur in groups and may fill a cell process or even the whole cell-body: they are not PAS-positive.

(3) The third form has scanty eosinophil cytoplasm and a shrunken pycnotic nucleus. The cytoplasm is PAS-negative.

The testicular histology of the last four embryo sizes is similar: central differentiation of the tubules and intertubular tissue is beginning to catch up on peripheral differentiation. Only typical interstitial cells persist; the pycnotic forms and those with eosinophil granules have largely disappeared. As in earlier testes, staining with haematoxylin and eosin or with PAS reveals transition forms between stellate mesenchymal cells and typical interstitial cells.

Results with Sudan black. The testes of the 8·1-cm and 9·7-cm embryos are alike (fig. 1, D). There are no sudanophil elements in the centre of the testis, where differentiation of the various constituents is incomplete. Elsewhere

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FIG. 1 (plate). A, testis of 2·8-cm embryo. 5 μ, PAS. The gonad consists entirely of stellate mesenchymal cells.

B, testis of 24-cm embryo. 5 μ, PAS. Note the absence of Schiff-positive material in the cytoplasm of the interstitial cells.

C, testis of 17·5-cm embryo. 5 μ, haematoxylin and cosin. Some epithelioid interstitial cells have eosinophil cytoplasmic granules.

D, Testis of 9·7-cm embryo. 10 μ, Sudan black. The centre of the gonad is poorly differentiated: the radial arrangement of the tubules in the remainder is conspicuous.

E, testis of 24-cm embryo. 10 μ, Sudan black. Note the lack of interstitial lipids in the centre (*c*) of the gonad, the intense sudanophilia of the paracentral (*p*) interstitial cells, and the radial arrangement of interstitial cells in the intermediate (*i*) zone.

sudanophil interstitial cells are arranged radially in columns of 2 to 8 cells between the seminiferous tubules. The abundant cytoplasm of the individual cells contains numerous minute sudanophil particles: the ground cytoplasm and nuclei are not coloured. Nondescript cells, intermediate in size and shape between typical interstitial cells and mesenchymal cells, are also visible: their cytoplasm contains a variable number of sudanophil inclusions.

The testes of the 17·5-cm, 21·3-cm, 24-cm, 31-cm, and 33-cm embryos resemble one another. The tubules are well defined in the centre of the organ and the interstitial cells may be divided into 4 groups or zones. In the first or peripheral zone, which lies just below the tunica albuginea, the cells are arranged in clumps and have sudanophil cytoplasmic granules. The cells of the second or intermediate zone (fig. 1, E) are histologically similar but are arranged in radial columns. In the third or paracentral zone, which circumscribes the area of newly-defined seminiferous tubules, the cells are coloured very deeply indeed by Sudan black and their cytoplasm is packed with dust-like black particles. In the centre of the testis the last or fourth group of interstitial cells is imperfectly differentiated; these 'immature' cells have no sudanophil cytoplasmic inclusions. At this stage, however, it will be noted that 'typical' interstitial cells are visible in the centre of the testis in the corresponding material stained with haematoxylin and eosin.

The testes of the 36-cm and 40-cm embryos only differ from the above description in so far as the cells of the central zone have acquired sudanophil inclusions and are not distinguishable from those of the paracentral zone.

Results with the plasmal test. At no time do the interstitial cells contain Schiff-positive lipids (plasmalogens, acetal phosphatides, and possibly steroids); nor is there any evidence of plasmalogens in the Sertoli cells.

DISCUSSION

Most recent workers agree with Gillman's (1948) statement that the testicular interstitial cell arises from a spindle-shaped mesenchymal precursor. In the foetal sheep testis, while many of the peripheral interstitial cells make their appearance during a period not covered by the present available material (i.e. between the 2·8-cm and the 8·1-cm stage), the more centrally placed cells can be seen to arise by transformation of mesenchymal elements derived from the undifferentiated central portion of the gonad. A similar, centrally placed portion which differentiates more slowly than the remainder of the gonad has been described in the foetal rat testis (Roosen-Runge and Anderson, 1959). On the basis of the PAS and Sudan tests, one can easily recognize that histochemical and histological metamorphosis of the interstitial cell from a mesenchymal precursor does occur.

In the intertubular spaces of testes traversing the inguinal canal (crown–rump length 17·5 cm, 21·3 cm, and 24 cm), there are three types of interstitial cell. While the occurrence of the typical hexagonal epithelioid cell is to be expected, the significance of the shrunken pycnotic forms and of the clumps of eosinophil granules in otherwise cytologically typical cells is problematical. Abnormal shrunken cells are also to be seen in the postnatal mouse testis (Baillie, 1958); they may represent atrophic or regressive forms of interstitial cell.

In man and the horse (Gillman, 1948) foetal interstitial cells reach a developmental maximum at or about mid-term. Thereafter the interstitial tissue undergoes a reduction whose occurrence and timing have not been satisfactorily explained. By contrast, in the rat (Roosen-Runge and others, 1959), in the rabbit (Allen, 1903), and in cattle (Bascom, 1923) similar interstitial cell maxima do not occur until birth. The ensuing interstitial tissue regression in these animals has been ascribed to deprivation of maternal oestrogens and gonadotrophins. In the present material there is no histological or histochemical evidence, at any stage, of significant interstitial cell degeneration; the post-natal fate of these cells in the sheep has not been pursued.

The cytoplasm of the interstitial cells of the foetal sheep contains no glycogen, glycolipid, or glycoprotein masses, i.e. no PAS-positive granules. This contrasts with the cytoplasm of the interstitial cells of poikilotherms (Cavazos and Melampy, 1954), which contains glycogen, and with that of adult homiotherms, which contains diastase-resistant polysaccharide / protein complexes (Montagna and Hamilton, 1952). On the basis of these findings it appears that glycogen is entirely absent from the foetal and adult homiothermal interstitial cell; the prominent red, refractile, diastase-resistant granules to be seen in the interstitial cell of the adult ram (Cavazos and Melampy) must make their appearance some time after birth.

In common with the sparrow, chaffinch, greenfinch, mouse, and Leghorn cockerel (Lofts and Marshall, 1956), rat (Lynch and Scott, 1951), deer (Wislocki, 1949), and man (Mancini, Nolazeo, and Balze, 1952) the interstitial cells of the foetal sheep contain sudanophil material. The material occurs in the form of very fine droplets which are only a fraction of the size of the corresponding droplets in interstitial cells of mice (Baillie, 1958).

No Schiff-positive lipids occur in the interstitial cell of the foetal sheep. Similarly, these lipids are absent from the interstitial cells of neonatal mice, cold-stressed mice (Baillie, 1958), the adult rat (Albert and Leblond, 1946), and the adult deer (Wislocki, 1949). It would appear that Schiff-positive lipids do not occur in the foetal testes of species so far investigated; they are present in large amounts in the interstitium of most adolescent animals so far studied; they are usually present in reduced amounts in the corresponding adult testes, although some adult testes contain no demonstrable plasmalogens. In addition to age and species variation, climatic factors also influence the amount of interstitial cell plasmalogen (Baillie, 1958).

While the above histochemical findings suggest that interstitial cell androgens are present in small quantities, if at all, in the foetal sheep, androgen has been demonstrated in the testis of the foetal bull (Moore, 1950), and it has recently been shown (Price and Pannabecker, 1956) that the foetal rat testis *in vitro* produces demonstrable androgens. It is difficult to reconcile these observations with the classical view that the male secondary sexual organs require merely an initial androgenic stimulus for their differentiation, and that, for a time thereafter, their development is independent of the male sex hormone (Moore, 1950).

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The Fine Structure of Epithelium and Mesogloea in a Sea Anemone

By E. J. BATHAM

(*From the Portobello Marine Biological Station, Portobello, New Zealand*)

With two plates (figs. 1 and 2)

SUMMARY

Ultra-thin sections of mesenteries of a sea anemone provisionally referred to *Metridium canum* have been examined in the electron microscope. The flagella show the common 9:2 fibrous structure, the peripheral fibres fusing basally and appearing to run through continuously to basal corpuscle and banded rootlet. Arising from the epithelial surface, as well as flagella, are numerous palisade processes, some radiating from flagella bases. These extensive processes show internal fibrous structure. Mesogloeal fibres show strong banding, with a period of about 42 to 46 mμ.

INTRODUCTION

AN electron microscopic study has recently been carried out on the mesenteries of *Metridium senile* (Grimstone, Horne, Pantin, and Robson, 1958). The present paper reports the results of a brief examination of another acontiate anemone, provisionally referred to *M. canum* Stuckey. Certain features shown more strikingly in material of the latter anemone are here described.

The species of anemone considered in this paper occurs half-buried in sandy mud at or below low tide, its pedal disk attached to shell or pebble. It is a khaki-coloured, plumose species, with many hundreds of fine tentacles and no catch-tentacles. It agrees in many respects with the anemone Parry (1952) describes from Heathcote Estuary, Christchurch, which she tentatively refers to Stuckey's *M. canum*. But, after a systematic examination just made, Professor Cadet Hand is describing this anemone as a new genus and species of the family Acontiophoridae. This species has a column proportionately about twice as long as in *M. senile*. Its mesenteric retractor muscle is more strongly developed and innervated, and its rapid shortening response is faster than in *M. senile*.

MATERIAL AND METHODS

Freshly-collected specimens from Otago Harbour were fixed in summer (Jan. 1958) at the Portobello Marine Station, New Zealand. In accordance with the experience of Grimstone and others (1958), portions of mesenteries of expanded anemones anaesthetized in equal volumes of sea-water and isotonic $MgCl_2$ were pinned on small wax plates and fixed for 1 h in fresh, cold 1% OsO_4 in sea-water. After rinses in chilled sea-water, pieces about 0·5 mm square were cut and transferred to chilled 1% phosphotungstic acid

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for 24 h, washed, and taken through changes of ice-cold alcohols to 70% alcohol. I later embedded these in partially-prepolymerized 3 : 1 butyl / methy methacrylate, which was then polymerized overnight in an oven at about 56° C, sectioned them with a microtome like that described by Hodge, Huxley, and Spiro (1954), and examined sections in a Siemens Elmiskop I electron microscope. This was operated by Mr. R. W. Horne at the Cavendish Laboratory, Cambridge.

The material chiefly consisted of pieces of the middle of primary non-directive mesenteries, including retractor muscles. Most sections were cut longitudinally in a plane tangential to the mesentery surface; these yielded cross-sections of flagella and surface processes at various levels. A few sections were cut longitudinally at right angles to this to show surface structure in longitudinal section, and others were cut transversely through the mesenteries.

RESULTS

The mesenteries of sea anemones are sheets of tissue running in radially from the body-wall. Covering each surface of a mesentery is a sheet of flagellated epithelio-muscular cells. One surface bears a longitudinal muscle-layer, locally folded to form a stout retractor muscle. Between these two endodermal epithelio-muscular layers lies the fibrous mesogloea. The only cells present in the mesogloea are amoebocytes. Among the epithelio-muscular cells lie mucus-cells, amoebocytes, neurones, sensory cells, and nematocysts, which are not considered in the present paper. At its free, outer surface, each epithelio-muscular cell has a single flagellum. The cell surface surrounding the flagellum is not smooth, but shows an elaborately patterned structure, here to be described.

Fine structure of epithelial surface of mesenteries. The free surface of a musculo-epithelial cell, whether on radial or retractor surface, shows emerging from its centre a single, long flagellum. The flagellum, about 200 to 250 mμ in diameter, shows in transverse section the usual peripheral ring of 9 fibres enclosing 2 central ones (fig. 1, C), just as do most cilia and flagella of plants and animals so far examined (Fawcett and Porter, 1954; Bradfield, 1955;

FIG. 1 (plate). A, longitudinal section of mesentery in retractor region, tangentially shaving surface of epithelium. Surface portions of 3 epithelio-muscular cells appear at upper edge. Below, 3 flagella and the numerous palisade processes of other cells are cut transversely.

B, longitudinal section of flagellar base of epithelio-muscular cell of retractor muscle.

C–F, transverse sections of flagella, at levels indicated on B.

C, transverse section of free flagellum, showing 9 : 2 fibrous structure.

D, lower transverse section, shaving palisade processes.

E, 9 peripheral fibres of flagellum are fusing, and show radiating 'struts' just above the basal granule.

F, flagellum cut through hollow portion of basal granule. Peripheral fibres have fused, but the 9 'struts' radiating from them show sharply.

G, detail of palisade processes of surface of epithelio-muscular cell. Note double membrane and suggestion of fibrous structure in each 'bead'.

H, tangential section of surface of musculo-epithelial cell. Palisade processes radiate from flagellum in centre.

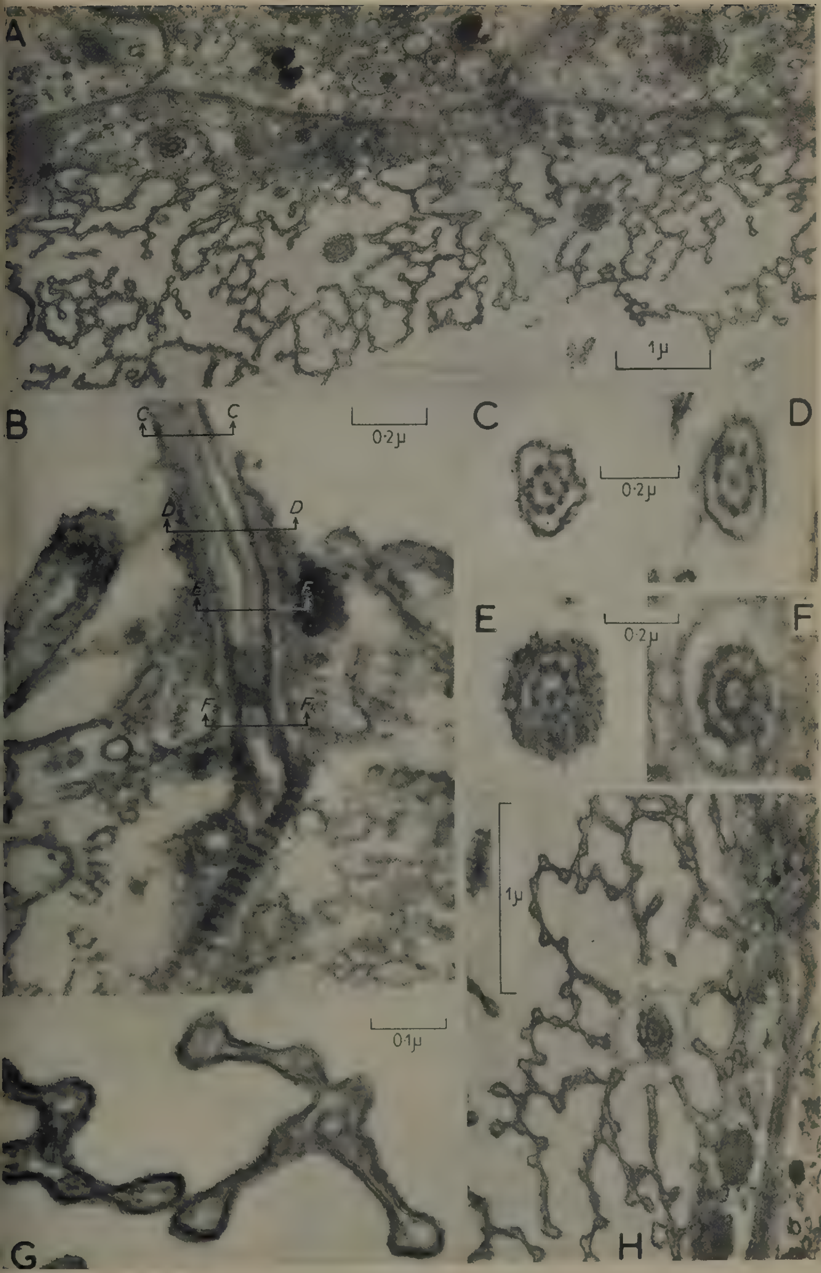

FIG. 1

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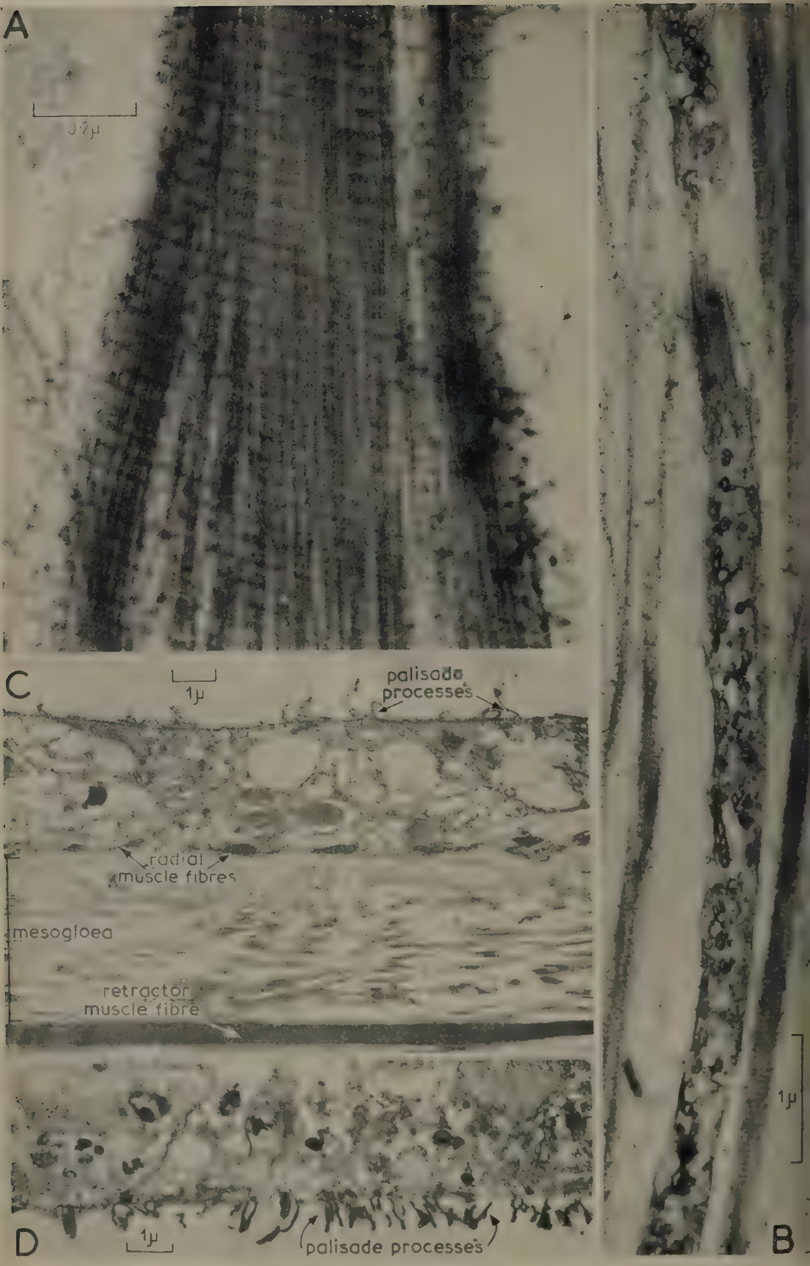

FIG. 2

E. J. BATHAM

Grimstone and others, 1958). Surrounding these fibres is a membrane continuous with that of the rest of the cell. As the peripheral fibres are followed towards the base of the flagellum, they converge slightly and fuse to form a cylinder, from which 9 struts run on to the surrounding flagellar membrane (fig. 1, B, E). The two central fibres, on the contrary, cease abruptly. Just below this, the cylinder formed by the outer filaments is closed by electron-dense material. This seems to represent the top of the basal corpuscle, which is hollow at a slightly lower level (fig. 1, B, F). From here, apparently, the fused cylinder running in from the peripheral fibres passes down into the flagellar rootlet. This rootlet was probably, but not certainly, detected in tangential (cross-)sections, and was shown for any distance in only a single longitudinal sagittal section (fig. 1, B). Here a rootlet runs in for 0·7 μ, diverging from the exposed flagellum at a slight angle. So far as the evidence goes, each flagellum appears to have one rootlet.

The flagellar rootlet in fig. 1, B shows a striking banding. The main bands lie approximately 42 mμ apart in this single example so far observed, while a secondary band lies closely below each main band. This banding period is less than the 52 mμ recorded by Grimstone and others for *M. senile*, or the 67 mμ recorded by Goreau and Philpott (1956) for the coral *Mussa angulosa.* But these differences are of uncertain significance, as Fawcett and Porter record a range of period from 50 to 72 mμ in the banding of different rootlets in one species of mollusc, *Elliptio complanatus.* All these flagella from diverse species agree, however, in showing striking periodic banding of the rootlet.

There appears in these *Metridium canum* flagella to be continuity from peripheral fibres to basal corpuscle to rootlet. In this they agree with flagella of *M. senile* (Grimstone and others, 1958), *Pleurobrachia* (Bradfield, 1955), and mouse oviduct (Fawcett and Porter, 1954); whereas the last authors found that in the molluscs and Amphibia examined, the ciliary fibres and basal corpuscle seemed to be separate structures.

The flagellar base, at the level of its basal corpuscle, is surrounded by a crater (fig. 1, B, F). This seems, in *M. canum* sections so far examined, smaller and less sharply defined than that of *M. senile* (Grimstone and others, 1958). Within the crater, outside the cell membrane, a sparse, amorphous substance seems regularly present.

Both around the crater of the flagellum and over the rest of the exposed epithelial surface are striking processes. In longitudinal sagittal section (fig. 2, D) these suggest microvilli, such as are described by Fawcett and Porter in *Elliptio*. However, when they are examined in sections tangential

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FIG. 2 (plate). A, longitudinal horizontal section of mesogloea in retractor region of mesentery, showing banding of mesogloeal fibres.

B, longitudinal horizontal section of mesogloea in retractor region of mesentery, showing elongated amoebocyte among banded mesogloeal fibres.

C, longitudinal section of mesentery, from radial surface (uppermost) through mesogloea to beginning of retractor muscle.

D, same section as last, epithelial surface of epithelio-muscular cells of retractor. Note greater density of palisade processes at epithelial surface in D.

to either surface of a mesentery, they are seen to be connected sheets of tissue rather than separate villi. They appear beaded in section, as a palisade would be (fig. 1, H). Inside each 'bead' or pillar are several fibres, suggestive of rudimentary flagellar structure (fig. 1, G). Externally these palisade-like sheets are bounded by a double membrane (fig. 1, G), continuous with the rest of the cell membrane. These processes are dense and about 0·8 μ high on the retractor face epithelium, less dense and slightly shorter on the radial face (compare fig. 2, D with fig. 2, C, retractor and radial faces of *same* section). The thickness of each sheet or process is much the same at its base as near its outer surface, as can be seen in fig. 1, A, by following the processes from solid tissue outwards. Their diameter through the centre of a swelling is about 80 mμ; between swellings about 20 mμ. These palisades are not simple sheets, but form an elaborate pattern partially related to the flagella. Near its base and just outside its crater, a flagellum is encircled by the inner margin of a ring of processes. Slightly above, the inner ring breaks, leaving about 9 processes radiating away from the flagellum, whose 9 peripheral fibres are fused at this level (fig. 1, H). A little farther out, the free flagellum stands in a space, while the radiating processes may fuse and loop together, losing the regularity of their pattern (fig. 1, A, centre). Then quite abruptly they cease, only the more widely scattered flagella protruding beyond.

In one specimen, the lateral membranes between adjacent cells protruded above the level of the processes. But elsewhere the opposite state prevailed.

Microvilli have been described on the surfaces of various ciliated epithelial cells, e.g. in *Mytilus* by Bradfield, in *Elliptio* gills and human Fallopian tubes by Fawcett and Porter, and in *M. senile* by Grimstone and others. Goreau describes microvilli radiating from flagellar bases in the coral *Mussa angulosa*, which appear closer to *Metridium canum*'s surface processes than outgrowths figured on other epithelial surfaces. In *M. canum* they appear better developed than the corresponding processes in *M. senile*, though there is the possibility that the state of the animals when fixed or other factors might influence these fine processes. Further, it is not known how these processes behave in the very long anemone *M. canum* when the mesenteries are extremely expanded or contracted, the material described being in a state of moderate expansion. They do seem, however, to be more elaborate in pattern and of more structural complexity than processes so far described on surface epithelia in other animals.

Such processes very greatly increase the free surface area of epithelial cells in *M. canum* mesenteries. Until more is known of similar epithelial processes in other species and other regions of this species, it would be unprofitable to speculate on their functional significance.

Banding of mesogloeal fibres. Mesogloeal fibres in *M. canum* have shown little structural detail under the light microscope. Under the electron microscope, however, a striking banding is apparent. In *M. canum* the main bands were spaced about 42 mμ apart in one preparation (fig. 2, A), about 46 mμ in another. There is also a faint secondary band about half-way between each primary band. The bands run across each of the many mesogloeal fibres in a group.

Although actinian mesogloea is generally regarded as collagenous (Rudall, 1955), the period of this banding in *M. canum* stands somewhat far away from the typical collagen figure of about 64 mμ. It also appears distinct from the period of 22 to 25 mμ found by Grimstone and others in the mesenteric mesogloea of *M. senile*.

Amoebocytes occur both in the epithelium and in the mesogloea. Fig. 2, B shows within the mesogloea an elongated amoebocyte lying alongside and in intimate contact with mesogloeal fibres.

The writer thanks the Zoological Laboratory and the Cavendish Laboratory, Cambridge, for the generous facilities she enjoyed while on sabbatical leave there. She especially thanks Prof. C. F. A. Pantin, F.R.S., who has extensively guided this work; Dr. A. V. Grimstone, who taught her how to cut ultra-thin sections; Mr. R. Horne, Assistant Director of Research in Electron Microscopy at the Cavendish Laboratory, who took most of the micrographs; and Miss J. Lawrence (Cavendish) who took those for figs, 1, B and 2, C, D.

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The Nerve-net of the Sea-Anemone *Metridium senile*: the Mesenteries and the Column

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With eight plates (figs. 2–4 and 6–10)

SUMMARY

The actinian nerve-net has been examined in the mesenteries and column of *Metridium senile* (L.) after staining with silver and with methylene blue. Modified staining methods are described. The synaptic nature of the junctions between bipolar nerve-cells, of their expanded terminations over the muscle-field, and of their contacts with the neurites of sense-cells is reviewed. The neurites always run in the space between the epithelium and underlying muscle. They follow the distribution of the main contractile systems, including the passage of circular fibres beneath the mesenteries. The richer innervation of the retractor surface of a mesentery compared to the radial is correlated with the ability of this hypertrophied muscle to contract rapidly. The distribution of nerve-cells and sense-cells in the mesenteries and column is related to physiological evidence concerning the through-conduction pathways, facilitated and slow contractions, and other aspects of the behaviour of *Metridium*. It is concluded that although features such as reciprocal inhibition in the column are still unexplained, there is as yet no histological or physiological evidence for double innervation of the muscles in this anemone. The terminations of sensory neurites, on muscle-fibres or elsewhere, have not yet been seen in any actinian.

INTRODUCTION

COELENTERATES have long been regarded as primitive metazoans in which the nervous system is found in its most elementary form. The organization of the nervous system, and indeed that of any coelenterate tissues, is, however, by no means simple, and the behaviour of these animals may be quite complex. In the case of sea-anemones and medusae, many of the special features of behaviour have been analysed in terms of neuro-muscular function, and physiological properties have in turn been related to the anatomical pattern of the nervous system, muscles, and sense-cells. It is not always easy to recognize these elements histologically, however, and in the past nerve-cells especially were often confused with other structures. As a result, the various published accounts do not give a consistent picture of the nervous system. The different kinds of evidence available have been reviewed by Pantin (1952), and it can be shown unequivocally that in anemones at least a synaptic nerve-net is present: the word 'synapse' is here used with its bare anatomical definition of a region of contact between independent nerve-cells, and without implication of any structural or physiological characters which hypotheses suppose them to possess in particular species of animals.

In such a synaptic nerve-net the different kinds of nervous connexions and

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neuromuscular endings are of fundamental importance, and it is proposed to give here a detailed account of them. In studying them histologically, the greatest care has to be taken to eliminate gross artifacts; nevertheless, careful examination of the artifacts encountered during this work has helped not only to clarify the true nature of the nerve-net, but also to explain many of the discordant results of other workers. This will be considered in a subsequent paper.

Histological methods for demonstrating nerve-cells generally include metallic impregnations of various kinds and especially silver staining, and vital staining with methylene blue. Experience has shown that none of these methods is invariably successful, even for one and the same material. In the case of *Metridium senile* (L.) it has proved necessary to modify certain standard procedures in order to stain the nerve-net at all. The following results are derived mainly from preparations of whole mesenteries. It is proposed first to give an account of the structure of the nerve-net and sensory systems that our methods show. In the subsequent paper we shall consider the criteria of identification of structures as nerve-cells and the various artifacts with which they may be confused. Existing data about the nerve-net can then be critically considered.

METHODS

Anaesthetization and fixation. Whatever method of staining is used it is necessary to fix the animal in such a manner that the fixative has rapid access to all parts. The anemone should be well expanded before fixation. That is best ensured by keeping a healthy animal in cool clean sea-water in the dark. It should then be anaesthetized. The best anaesthetic is a mixture of equal parts of sea-water and 0·36 M $MgCl_2$ (= $7\frac{1}{2}\%$ $MgCl_2.6H_2O$) which is about isotonic with sea-water. The animal can withstand at least 24 h in this mixture and recover completely.

Our procedure is as follows: The $MgCl_2$ solution is gently added to a dish containing an expanded anemone. In about 20 min response to touch has vanished. $MgCl_2$ sea-water solution is pipetted down the throat, and the anemone thus kept gently inflated. Better still, it is continuously inflated under a pressure of about 2 cm water from a funnel containing the Mg mixture, by way of a hypodermic needle pushed through the lower part of the body-wall. The animal will then slowly expand. Inflation should not be allowed to proceed too far or it results in distortion or rupture. Usually an animal 4 or 5 cm across the pedal disk is ready for fixation or dissection in about 1 to 2 hours.

For fixation, the animal is lifted entire into the fixative; and by means of a 50-ml pipette large quantities of fixative are immediately and continually injected gently down the throat to distend the body, for several minutes. Alternatively, the anaesthetized and inflated animal may be laid in a dish and opened. Thin sheets of paraffin wax can then be slipped beneath a mesentery or beneath other parts of the body. These parts can be pinned with hedgehog spines (metallic pins were avoided) to the wax sheet and dissected free. In this state they can be examined alive, with or without vital staining; or they can be transferred to fixative. A glass window may be left in the wax sheet to facilitate *intra vitam* observation.

Silver staining. After trying out several methods which had been reported to stain coelenterate nerve-cells, it was found necessary to carry out lengthy trials of existing procedures before the nerve-net of *Metridium* could be stained at all. The method finally developed (by E. J. B.) is a modification of Holmes's procedure (1942, 1943) which he found successful for peripheral nerve in vertebrates and other animals. It has proved successful with whole mesenteries, the body-wall and the disk of *Metridium.* An extended anemone which has been anaesthetized and fixed provides thin mesenteries which may be handled easily as whole mounts.

The best fixative was: Picric acid, sat. aq. 3 vol., with commercial formalin 1 vol. Aqueous Bouin also sometimes gave good results.

After fixation in the way described above, the animal is left in the fixative for some 24 to 48 h. It is then transferred directly to a large volume of 70% ethanol which is pipetted down the mouth. The ethanol is changed several times, during which the anemone is opened. Later, mesenteries are cut out and lightly tied to microscope slides with threads as flat preparations. Alternatively the tissue may be embedded in paraffin and sectioned as usual.

The whole tissues tied to slides are treated as follows:

(1) Transferred from 70% ethanol to distilled water (10 min).

(2) Into 20% $AgNO_3$ in the dark, 1 h.

(3) Wash in distilled water, several changes.

(4) Place in an oven at 35 °C for 24 to 48 h, in the following impregnating solution, freshly made up from 4 stock solutions:

$AgNO_3$, 1% in distilled water	2 ml
Pyridine, 10% in distilled water	2 ml
Boric acid (0·2 M, or 12·4 g.p.l. H_3BO_3)	14 ml
Borax (0·05 M, or 19·0 g.p.l. $Na_2B_4O_7$, $10H_2O$)	6 ml
Distilled water	180 ml

This gives a 1 : 10,000 solution of $AgNO_3$, buffered at about pH 8.

(5) After impregnation, rinse each slide in distilled water.

(6) Place for 2 or 3 min in fresh reducer solution:

Hydroquinone	1 g
Sodium sulphite, anhydrous	5 g
Distilled water	100 ml

(7) Wash for about 3 min in running water.

(8) Place for 3 to 10 min in $AuCl_3$ solution (0·2%, usually with 3 drops glacial acetic added per 100 ml. The solution lasts several weeks).

(9) Rinse in distilled water for about 10 sec.

(10) Place in fresh 2% oxalic acid in a Petri dish on the stage of a microscope. In successful preparations nerve-fibres begin to show in a minute or two.

(11) When the nerve-fibres stand out clearly (after 1 to 10 min in the oxalic acid in successful preparations), rinse in distilled water for 10 sec.

(12) Fix in fresh 5% 'hypo' for 5 min.

(13) Wash in running tap-water for 10 min.

(14) Place mesentery under cigarette paper on a slide to keep flat.

(15) To ensure flexibility of preparation for mounting, dehydrate rapidly ($\frac{1}{2}$ to 1 min each) in a long range of ethanol mixtures, and transfer to methyl benzoate. Mount directly in balsam under a thin, weighted coverslip.

In some preparations, one epithelial layer was stripped off with sharpened watchmaker's forceps. This was done in a dish of methyl benzoate (stage 15) under a dissecting binocular.

Methylene blue. In studying a particular kind of cell by methods which are not wholly specific, it is necessary to compare information from a variety of sources. In practice this involves the use of several histological methods on the same material and, if possible, the examination of living tissues. The chief method we used in *Metridium* alternative to silver staining was vital staining with reduced methylene blue by Unna's method (Pantin, 1948) on fresh mesenteries. In successful preparations this stain reveals both the nerve-net and the sensory system, and many results obtained with Holmes's silver method have been confirmed.

A satisfactory method was developed (by E. A. R.) for the fixation of preparations stained with methylene blue in the following way. Only thin sheets of tissue from well-extended animals can be used, because preparations darken after fixation.

(1) Extend anaesthetized tissue on a wax plate as already described. Stain with reduced methylene blue in magnesium sea-water.

(2) Wash briefly in stain-free magnesium sea-water.

(3) Fix in Heidenhain's Susa, 3 h to overnight. Specimen is now pink.

(4) Pass directly to fresh 1% phosphomolybdic acid: leave in this until specimen has assumed a turquoise blue colour. (This usually takes less than 30 min but may take longer.) Colour is now fast.

(5) Counterstaining may proceed at this stage.

Or:

Wash in distilled water a few minutes.

(6) Dehydrate:

95% ethanol with iodine—95% ethanol—two changes of absolute ethanol and xylene. Mount in Canada balsam. The times depend on the specimen, but usually leave in iodine-ethanol and other ethanols for 20 min or more each.

The preparation subsequently darkens. While this is no disadvantage with thin tissues, it renders the method useless for preparations thicker than about 40 μ. The dye is fixed in a granular state, but the fixation of the tissues is good. The preparations survive for at least several years. Because of their thickness, preparations of body-wall stained with methylene blue were fixed with ammonium molybdate; the preparation shown in fig. 9 was fixed in a cold 8% solution and then washed in changes of cold distilled water for several hours each. After blotting gently it was dehydrated in absolute alcohol, cleared in xylene, and mounted.

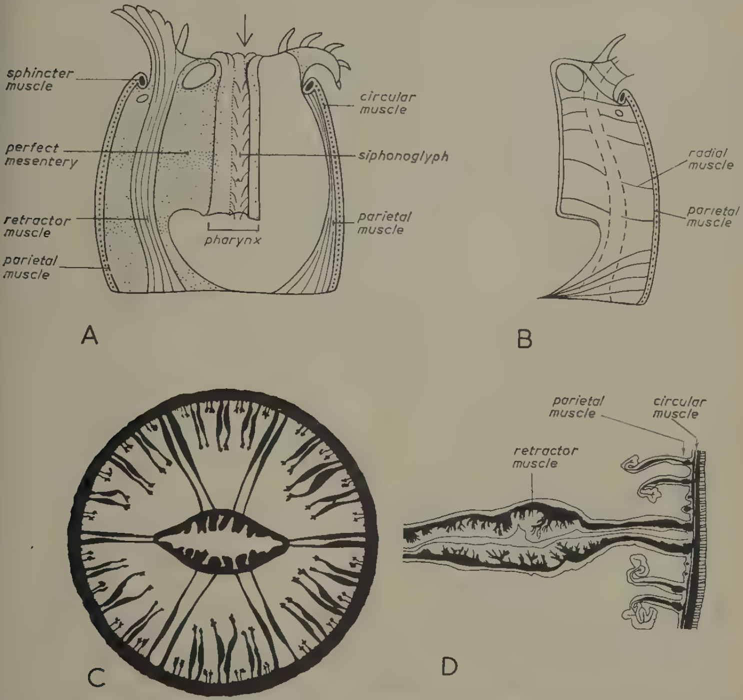

FIG. 1. Diagrams showing gross anatomy of *Metridium*. A, vertical section of whole animal, showing retractor surface of perfect mesentery (endocoelic view). B, radial surface of perfect mesentery (exocoelic view). C, transverse section at level of pharynx showing arrangement of mesenteries. D, part of C, more highly magnified to show position of retractor and parietal muscles.

RESULTS

Structure of the nerve-net

In this paper we are chiefly concerned with the structural relations of the nerve-net in the mesenteries and in the adjacent body-wall. Fig. 1 shows the anatomy of these structures.

From physiological evidence it is inferred that tracts of nervous through-conduction run up the mesenteries of various actinians and serve the retractor muscle. Both silver and methylene-blue techniques show the main nervous system of *M. senile* to consist almost entirely of bipolar nerve-cells along this site. General illustrations of it in the region of the mesentery between the body-wall and the retractor have already been figured (Pantin, 1952).

Fig. 2 illustrates the rich nerve-net on the retractor surface of a perfect mesentery, but in this case over the edge of the retractor muscle-sheet, the folds of which can be seen underlying the nerve network. This preparation was vitally stained and fixed by the Susa/phosphomolybdate method. As in silver preparations the network of bipolar cells is clearly visible, but in addition simple sensory cells stained with methylene blue are seen.

Each mesentery of *Metridium* consists of two layers of endoderm with mesogloea between. The endoderm is built mainly of musculo-epithelial cells (Hertwig and Hertwig, 1879). The outer epithelial parts of the cells form a continuous pavement, and each outer part is connected with a fibre of the underlying muscle-sheet by a neck of cytoplasm. The cytoplasmic necks are separated from each other by an intercellular space (Robson, 1957; Grimstone and others, 1958). It is in this intercellular space that the nerve-fibres run. That this is the site of the nerve-net is shown by transverse sections, such as fig. 3, A, B, by careful focusing in both methylene blue and silver whole mounts, and by the maceration methods of the Hertwigs (1879). In the latter, macerated tissues are brushed so as to remove the outer parts of the epithelium, leaving both nerve-cells and sensory cells adhering to the underlying muscle-sheet in contact with the mesogloea. The preparation is later stained with borax carmine. Unstained living nerve-cells can be seen with a water immersion objective and are between the epithelium and the muscle (fig. 7, D).

In no case have nerve-cells been found except in this situation: they never occur in the mesogloea. The significance of this will be discussed presently. But the fact is easy to demonstrate over a wide area, not merely by careful focusing in preparations like that in fig. 2, but by stripping the tissue into its components. After the nerve-net in a mesentery has been successfully impregnated with silver, it is possible to strip away pieces of the muscle-sheets of the mesenteries together with the attached epithelial surface. This can be done on both sides of a mesentery, giving beautifully clear preparations of the nerve-net in its musculo-epithelium (fig. 6, A, B). The nerve-net is brought away intact within the epithelial surface above and muscle-sheet below. The mesogloea which remains can be seen to be without trace of nerve-fibres and to contain only amoebocytes. Mesogloea isolated from teased, macerated tissue shows the same thing.

Though the nerve-fibres run within the intercellular space, over much of their course they run nearer to the epithelial surface than to the muscle-sheet. This is most evident over the retractor muscle itself, where the nerve-net follows the smooth surface of the epithelium rather than following the repeated foldings of the muscle-sheet (fig. 3, B). Occasional nerve-fibres are

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FIG. 2 (plate). Methylene-blue preparation of a perfect mesentery showing the nerve-net of bipolar cells, and scattered sense-cells. This region is on the edge of the longitudinal retractor, and the folded arrangement of the underlying muscle-fibres is evident. (Fixed with Susa / phosphomolybdic acid.)

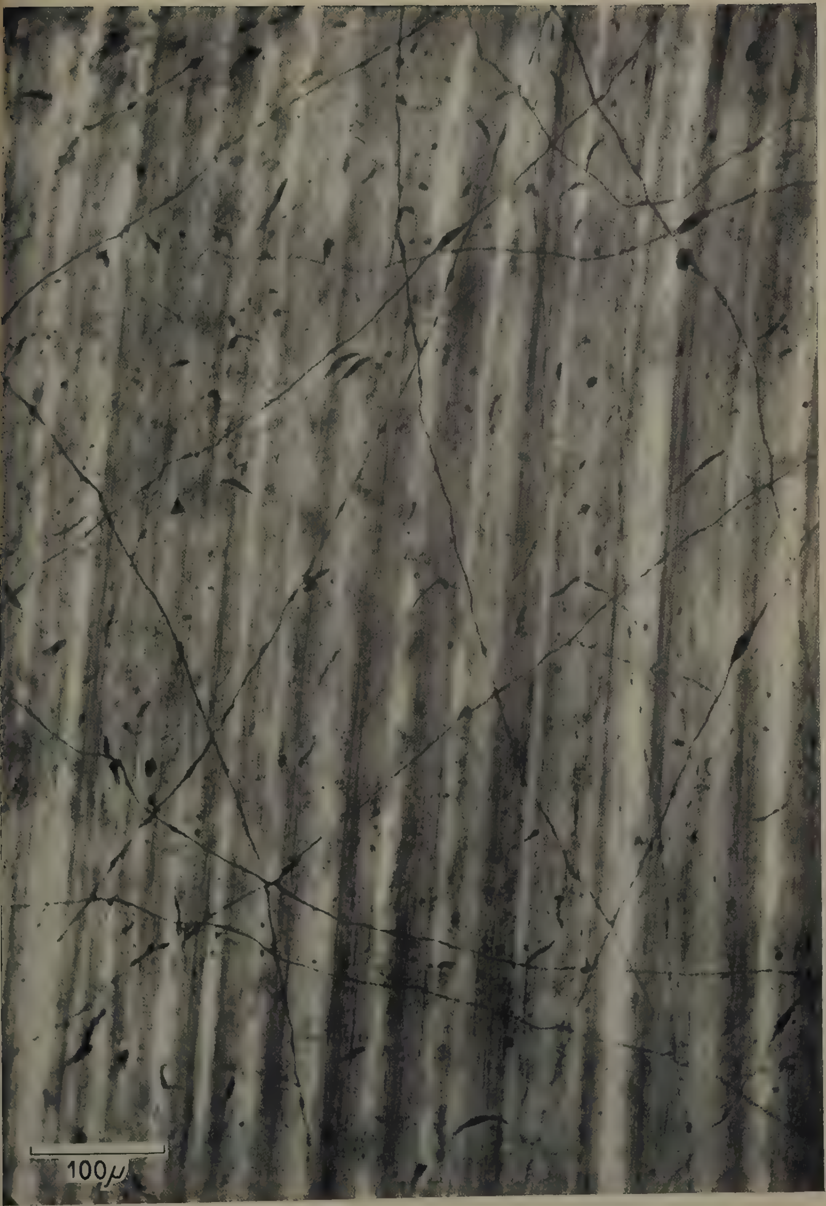

FIG. 2

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seen running between the muscle-folds. The nerve-fibres on the retractor surface of the mesentery tend to run parallel with the folds and fibre-direction of the muscle-sheet.

The density of the nerve-net varies considerably. Whilst it is very high in the tracts of the retractor surface, it is very low on the opposite radial surface of the mesentery. Fig 6, A, B shows silver preparations from approximately corresponding regions of the retractor surface (B) and the radial surface (A) of the same mesentery. The radial surface figured is actually more richly innervated than other parts of the same preparation, which show but one or no nerve axons in a field of this size; yet the sparseness of its innervation as compared with the retractor face is striking.

Fig. 4 shows the main feature of the net. The net consists of bipolar cells of various sizes. From each cell-body there usually arise two nerve-fibres or 'axons' (Pantin, 1952), some of which are shown in fig. 4, A. Because of the special meanings that have become attached to these terms in the vertebrate central nervous system, neither of them is wholly free from objection. Professor J. D. Boyd and Dr. A. Hughes have kindly advised us that the historically correct and most general term is 'neurite', and we shall therefore now refer to these processes as 'neurites'. The neurites attenuate as they recede from the cell-body. Usually they run a fairly straight course for long distances (millimetres). Consequently a field such as fig. 4, A is traversed by fairly straight neurites of different sizes. Where the neurites of different cells come in contact they make a synaptic junction of a kind already described (Pantin, 1952). The contact is not syncytial. Fig. 4, A shows contacts between neurites of equal and unequal size. It also shows crossed and parallel synapses. Very occasionally a neurite may rather abruptly change its direction (fig. 4, C).

The majority of the neurites can be seen to terminate in small hand-like expansions. A small example of this is present in fig. 4, A (see arrow). Such expansions in the majority of cases drop down from the general intra-epithelial level of the net and make contact with the fibres of the muscle-sheet.

Nerve-cell bodies and neurites

In life, whether or not stained with methylene blue, the cell-bodies are spindle-shaped. There is a large nucleus with a prominent nucleolus in stained preparations. The size of the nucleus seems to increase with the size of the nerve-cell, but much less so than does the volume of cytoplasm (fig. 4, B).

Silver preparations may show the spindle form of the cell-body, but commonly there is some distortion of the cell surface, and often flattening of the neurites, particularly in large nerve-cells. The fibrillar appearance of the neurites after silver staining has been noted and has been discussed elsewhere (Pantin, 1952). It may be seen in fig. 4.

Of greater interest are the size, the distribution, and the connexions of the neurites. The number of neurites of bipolar cells in a given area is much the greatest in the nerve-tracts of the large perfect mesenteries. It is smaller in the imperfect retractor faces; whilst the endocoelic faces (equivalent to the retractor faces) of the youngest mesenteries are not so richly supplied with nerve-cells—though more so than the radial surfaces of the mesenteries.

Thus there is a well-developed nerve-net over the retractors and adjacent parts of the retractor surface, and a more weakly developed net over the parietal muscles, which are best developed in the youngest mesenteries. In all the mesenteries, the net of the retractor face abruptly becomes much sparser where the mesentery joins the body-wall. A few of the neurites run on to the circular muscle-field of the column (p. 504).

The two faces of the mesenteries, the endocoelic and the exocoelic, differ in important ways on the retractor and the radial faces. A and B in fig. 5 (p. 496) are maps over about 6 sq. mm of all the nerve-cells in approximately equal and corresponding areas of the retractor and the radial faces from the large 'perfect' mesenteries. The region taken is over the through-conduction nerve-tract between the retractor and body-wall at about one-third of the distance from the oral disk to the foot.

As is obvious from the figures, the nerve-net on the retractor face is far richer than on the radial. Indeed, the relative number of cell-bodies in such an area on the radial surface is commonly smaller than in the example mapped. In this example the ratio of nerve-cell bodies retractor/radial is 49/6. Nevertheless, there is no great difference in the length of the neurites on the two surfaces.

Individual lengths of above 9 mm have been measured from end to end of some bipolar cells of the radial surface. It is not possible in a given field to follow and to measure a statistically large number of fine individual neurites for such great distances. And in any chosen field there are many neurites which are only partly included in it. But measurements on both faces commonly show average neurite lengths of 2 to 4 mm: that is, 4 to 8 mm from end to end of the bipolar cell. Table 1 gives small arbitrary samples of the distance from cell-body to the ending of the neurite over the muscle (p. 495) on the two faces of comparable mesenteries. They are taken from a small moderately expanded specimen 18 mm high by 19 mm in diameter.

In this case the average length of the measured neurites on the radial side actually exceeds that on the retractor, although the difference is not regarded as significant.

In contrast with their lengths, the nerve-cells on the two surfaces of the mesentery differ greatly in range of neurite diameter and in numbers. The neurites arising from the spindle-shaped cell-body decrease very slowly in

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FIG. 3 (plate). Silver-stained sections of mesenteries in the retractor region approaching the disk, showing neurites running in the space between epithelium and muscle-fibres. Note epithelio-muscular connecting strands.

A, endocoelic surface off the retractor. At least three neurites are seen above the longitudinal muscle-fibres.

B, endocoelic surface: two neurites are seen running just below the epithelium above pronounced folds of the retractor.

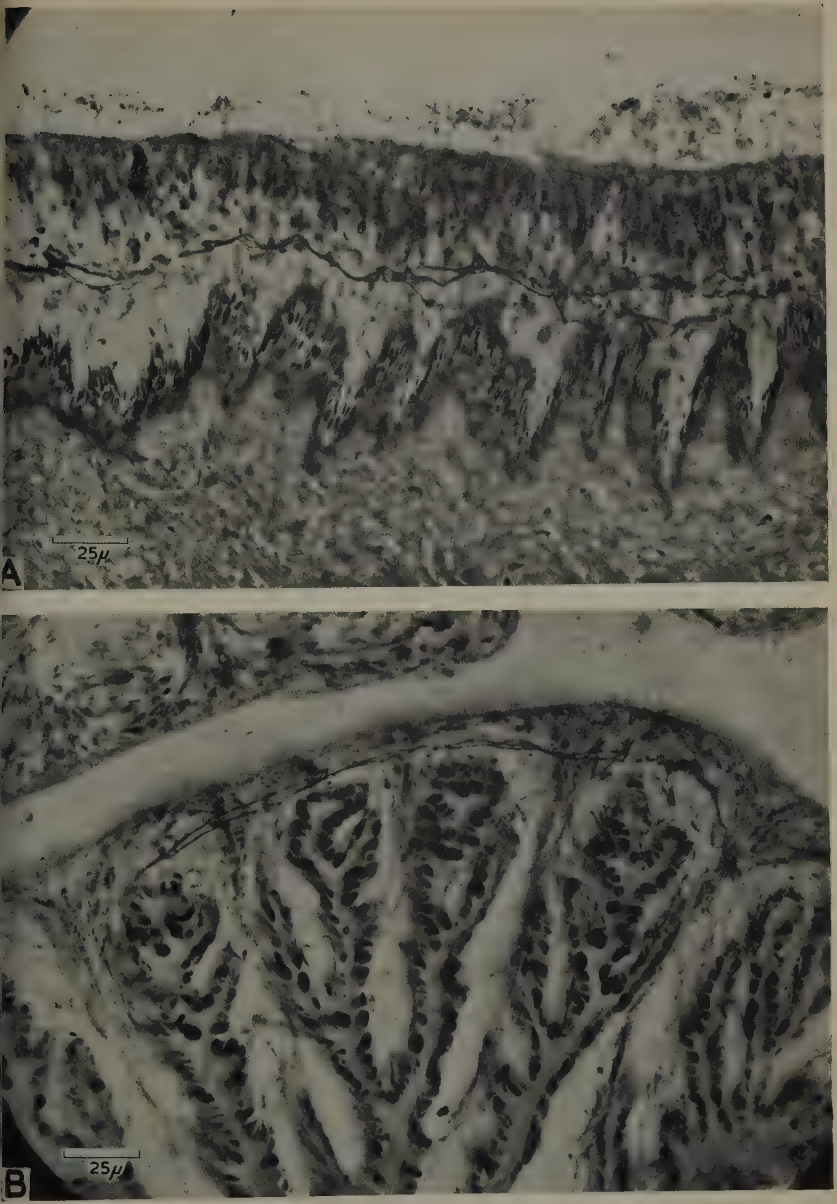

FIG. 3

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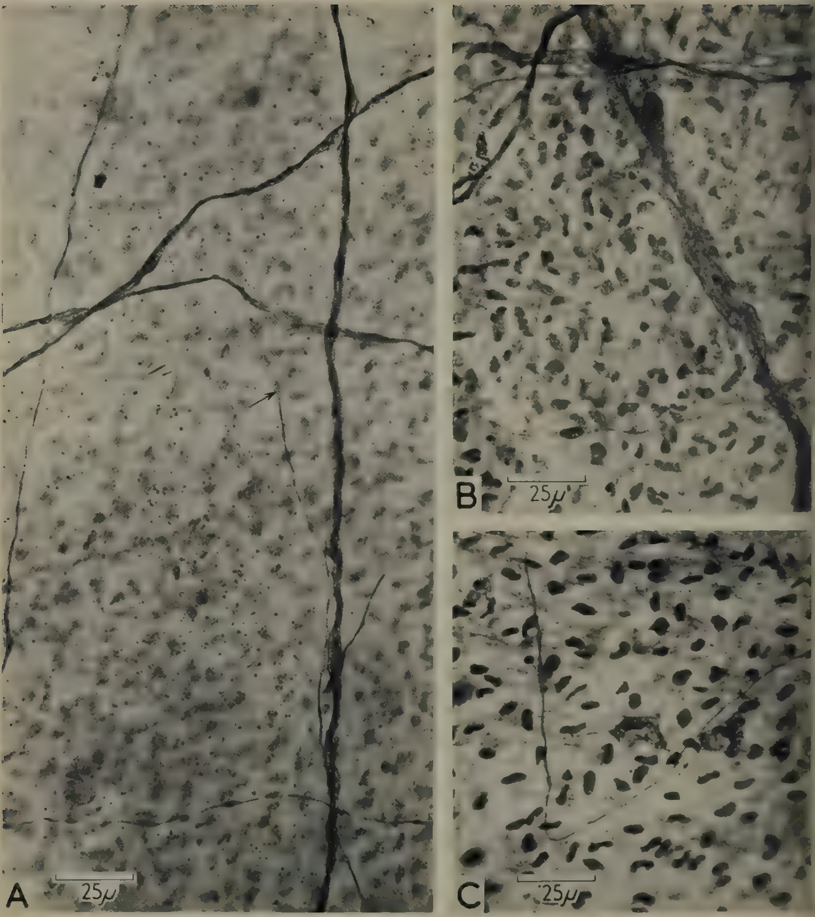

FIG. 4

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diameter until they are only a fraction of a micron in width. Just before they end, they may increase slightly in diameter. They terminate in hand-like expansions to be described later (p. 498). Similar appearances are sometimes to be seen in methylene-blue preparations, though methylene blue does not render the fine terminations of the neurites as clearly visible as does silver staining. The silver preparations are easier to measure, notwithstanding the fact that there is often more shrinkage of the cytoplasm, which leaves the neurite diameter somewhat irregular. Allowance can be made for this, however, and in table 2 the diameters of the neurites are shown as measured within about a cell-body's distance from the cell-body itself. Corresponding regions of the radial and retractor fields of the two mesenteries were examined in the same preparations as those measured in table 1.

TABLE 1

Length of neurite in mm	*Number of neurites in sample*	
	Radial face	*Retractor face*
2·0–2·4	–	1
2·5–2·9	6	3
3·0–3·4	4	1
3·5–3·9	3	1
4·0–4·4	3	6
4·5–4·9	1	1
5·0–5·4	–	–
5·5–5·9	–	–
6·0–6·4	1	–
Average length in mm . . .	3·7	3·2

TABLE 2

Diameter of neurite in μ	*Number of neurites in sample*	
	Radial face	*Retractor face*
0·0–0·9	2	6
1·0–1·9	8	24
2·0–2·9	–	9
3·0–3·9	–	4
4·0–4·9	–	–
5·0–5·9	–	1
Average diameter in μ	1·2	1·66

FIG. 4 (plate). Details of silver-stained preparations of whole mesenteries.

A, shows boundaries of junctions between neurites of bipolar cells, when they cross over or run in parallel. A thin neurite which makes several lateral contacts with a larger one ends in a small expansion which can just be seen (arrow at centre right).

B, size range of nerve-cells: central portion of a large bipolar cell including the nucleus contrasts with the slender nerve-cell at the lower border of the photograph.

C, change of direction occurs in occasional neurites. Two mucus-cells are seen in the centre of the field.

We at first had the impression that the neurite diameters in the retractor nerve-net might prove to be bimodal in distribution. But more critical observation and samples such as that given in the table did not bear this out. The few large neurites on the retractor face catch the eye of the observer and subjectively accentuate their apparent frequency. On radial faces of mesenteries, on the other hand, large neurites of more than 2 μ diameter have never been seen. There is a real hypertrophy of the nerve-cells on the retractor surface both in size and in numbers. The appearance of the retractor net is about the same over the retractor and between the retractor and the body-wall (compare figs. 2, 5, B, and 6, B). It is less dense between the retractor muscle and the inner limit of the mesentery.

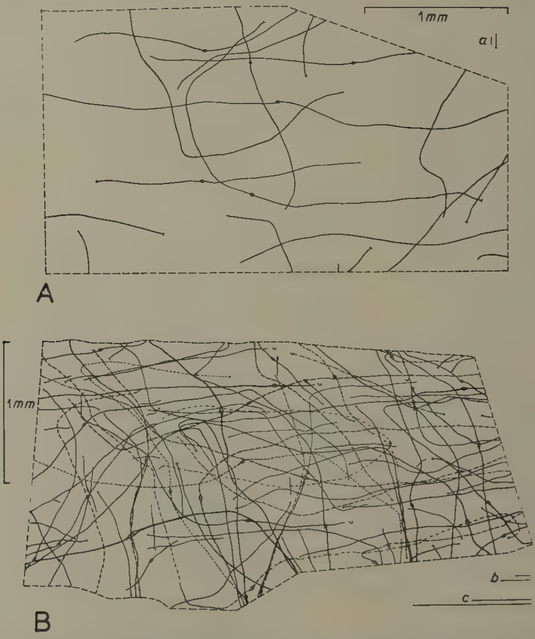

FIG. 5. From a silver-stained whole mesentery. A, map of nerve-net on the radial surface. Orientation and length range of muscle-fibres indicated at *a*. B, plan of nerve-net on corresponding area of retractor surface. Orientation and length range of retractor muscle-fibres shown at *c*, off-retractor fibres at *b*. Some neurites are indicated by broken lines for clarity.

Whatever their proportional distribution in relation to size, the number of

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FIG. 6 (plate). From the same anemone as fig. 5; A and B are from corresponding areas of another mesentery.

A, radial nerve-net. The dark objects are mucus-cells.

B, retractor nerve-net in a corresponding area of the mesentery.

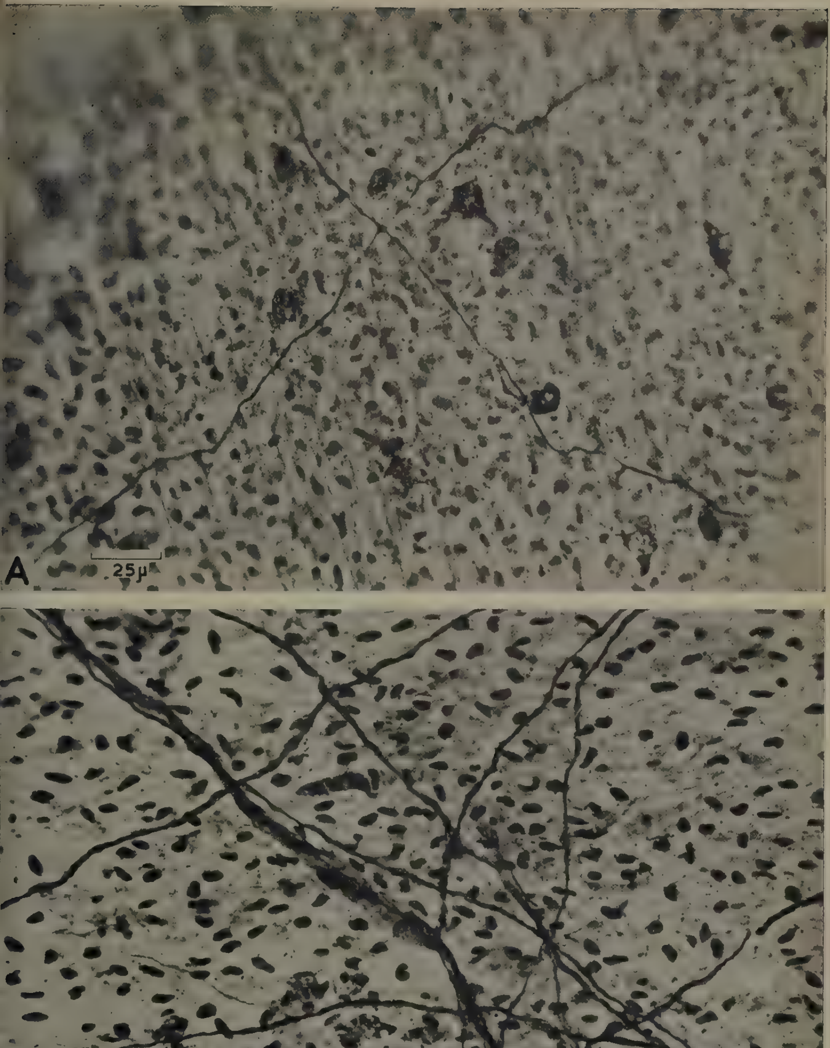

Fig. 6

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nerve-cells of all sizes, from small bipolars of less than 100 μ long up to giants of nearly 10 mm, is far larger on the retractor surface than on the radial.

As fig. 5 shows, on both sides of the mesentery there is some tendency for neurites to run vertically along its length. On the retractor surface, this takes the neurites along the direction of the muscle-fibres. On the radial surface, however, the neurites cross them at right angles and the number of muscle-fibres over which a neurite passes is increased. But it may be noted now that despite the innervation which is demonstrated by techniques we have used, large numbers of the radial muscle-fibres do not appear to be traversed by any neurite. The approximate range of size of the muscle-fibres on the radial muscle-sheet (lines *a*), and on and off the retractor muscle-sheet (lines *c* and *b*), is shown in fig. 5.

This tendency of neurites to run parallel to the retractor muscle on both sides of the mesentery is remarkable. The muscle can undergo a high degree of reversible extension. Its normal lengths at full extension and full contraction are as 5 : 1 (Batham and Pantin, 1951). Apart from their synaptic adhesions to each other, the position of neurites relative to the muscle-sheet is necessarily fixed by the forest of epithelio-muscular cytoplasmic connectives through which they run as they traverse the intercellular space system (compare Robson, 1957). It would seem, therefore, that neurites must be extensible or must be buckled on shortening, or be subject to both these effects. Observation of nerve-cells stained with methylene blue in the living mesentery show both these effects when the muscle is lengthened and shortened. The nerve-cells in a contracted mesentery are thicker than in extension, and in a mesentery which is allowed to contract a neurite is apt to become irregularly sinuous.

Apart from the tendency of neurites to run longitudinally up the mesentery, the organization of the nerve-net is not wholly random. Adjacent neurites frequently tend to run parallel and close together (fig. 4, A), with occasional synaptic contacts, often for distances of several hundred μ. Secondly, nodal junctions where three or more neurites cross each other seem rather frequent (fig. 2). Possibly junctions close to a cell-body are more frequent than might be expected.

The great majority of the mesenteric nerve-net cells are simple bipolar cells. Careful examination of an entire mesentery may, however, reveal one or two cells of the same sort and size with three neurites arising from the cell-body instead of two (as in fig. 6 in Pantin, 1952).

In the body-wall, there is a marked tendency for the neurites to run parallel with the circular muscle field (fig. 11). These neurites often run right through the base of the mesentery. They have not been seen to do so by penetrating the mesogloea, but pass through the tubes of muscle-fibres of the circular muscle field which pierce the attachment to the body-wall.

Some neurites of the body-wall on reaching the mesentery turn sharply and run parallel to it, providing contact with the mesenteric nerve-net (fig. 11). The muscle-fibres of the circular muscle-sheet are roughly between 100 and

500 μ long. As in the radial mesenteric muscle-sheet only a small proportion of the muscle-cells of the circular muscle are traversed by neurites.

Synapses

The neurites behave as though they had the property of adhering to other neurites and to the neurites of sensory cells. The contacts with other neurites have already been described (Pantin, 1952). They are not syncytial, but the one neurite 'wets' the other, with a definite boundary between them which stains with silver (figs. 4, A; 7, A, C, E). Methylene-blue preparations give exactly the same picture as silver preparations, even down to the local dilatation that takes place at these synapses, as in fig. 7, A, B. Contacts between fibres, large or small, running side by side are to be seen in fig. 4, A.

A second class of contact is shown in fig. 7, C. A fine 'twig' is given off by one neurite to another. As the figure shows, the silver staining boundary seen in the grosser synapses is also present in these twig junctions. Such junctions occur most often near the end of the neurite.

A third kind of synapse is occasionally found at the terminus of a neurite. An expanded end of the neurite extends a few twigs on to an adjacent neurite (fig. 7, F).

Nerve-endings

The vast majority of the neurites of the bipolar cells can be traced to expanded endings. Most commonly, as far as can be seen with the light microscope, these are in contact with a number of fibres of the muscle-sheet. In silver preparations their form varies from simple oval expansions (fig. 8, A, B) to plates with finger-like extensions on to the muscle (fig. 8, C, D) which may seem to have fine fibrils running from them (see fig. 12 in Pantin, 1952) and to branched structures (fig. 8, E, F). Examination suggests that some of the more extreme claw-like structures are to be found in those preparations in which fixation has caused shrinkage. It must also be borne in mind that some of the strand-like connexions with the muscles often found in silver preparations may perhaps be due to contraction in fixation or to deposition of silver on the musculo-epithelial connexions with which the end-plate comes in contact rather than on parts of the neurites. Occasionally the neurites end as a fine thread without any expansion. The fine terminal parts of neurites are more rarely stained in methylene-blue preparations than in silver preparations,

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FIG. 7 (plate). Details from whole mesentery preparations.

A, crossover junction between two neurites, showing contact boundary. Silver preparation.

B, similar junction in a methylene-blue preparation (fixed with Susa / phosphomolybdic acid).

C, lateral process from a fine neurite making synaptic contact with a larger one. Silver preparation.

D, unstained bipolar nerve-cell in a living mesentery.

E, broad region of contact resulting from crossover at cell-body. Silver preparation.

F, apparent termination of one axon on another. Silver preparation.

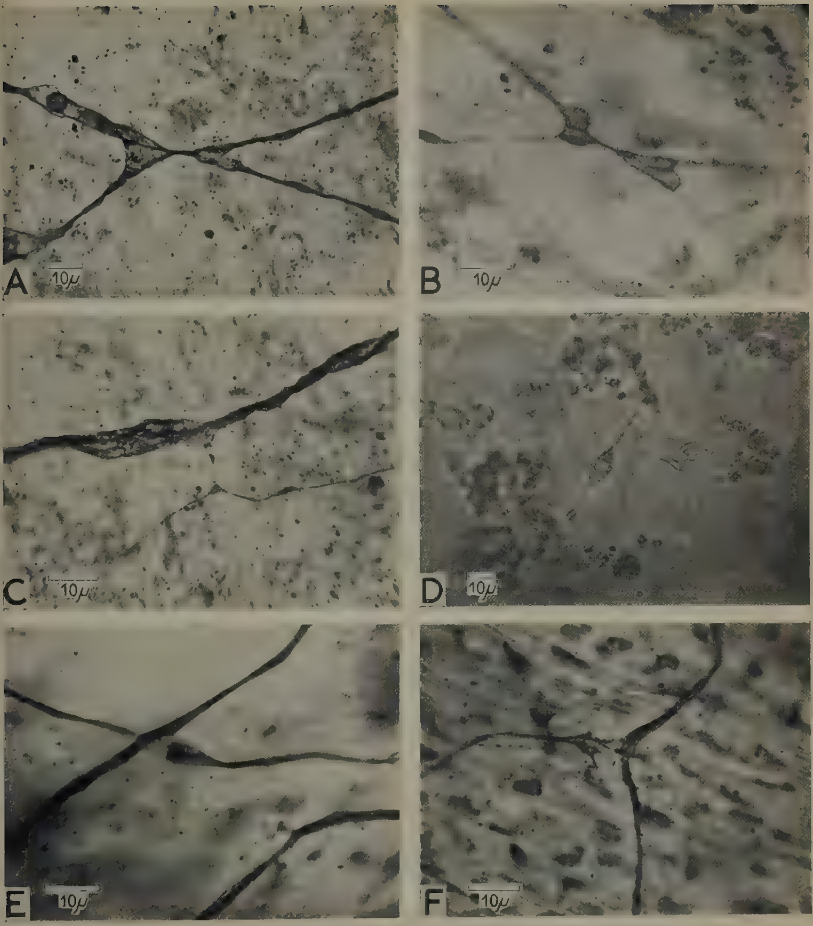

Fig. 7

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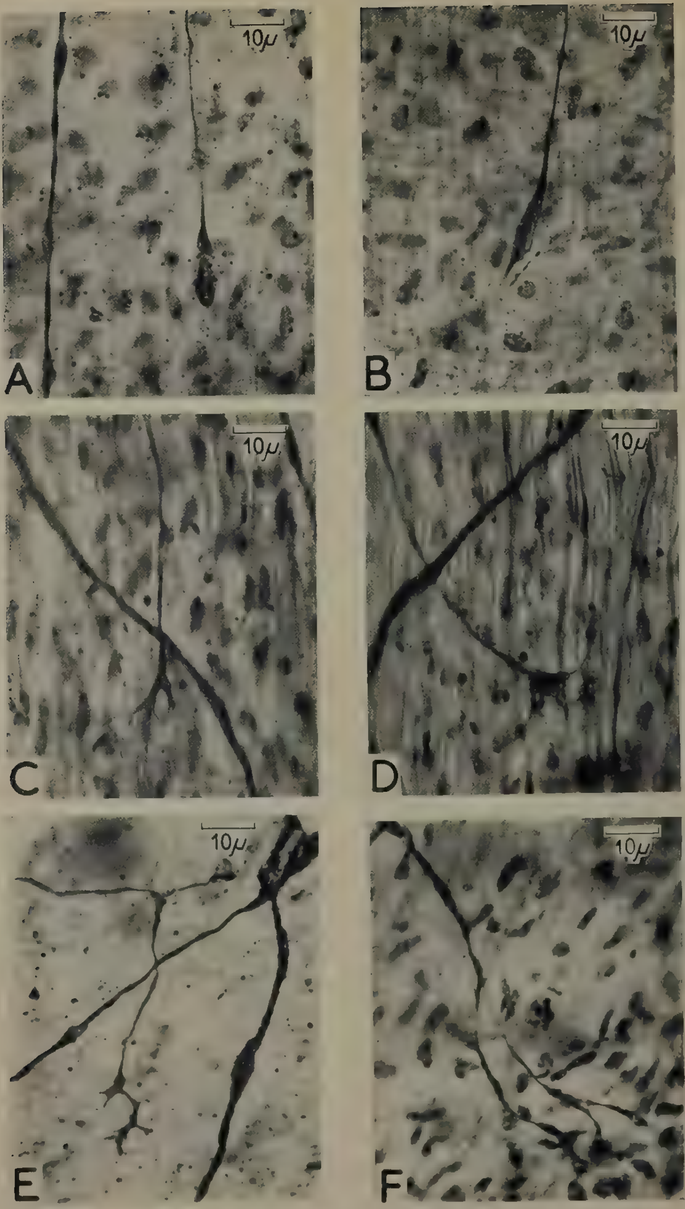

FIG. 8

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but occasionally in mesenteries stained with methylene blue some can be seen to end in expansions in contact with the muscle-fibres.

Pantin (1952) referred to these endings on the muscle as 'end plates'. It is clear that a morphological description such as we have given does not yet justify our going further and interpreting them as 'motor end plates' (Hoyle, 1957). It should be borne in mind that other kinds of contact between the nerve-net and epithelio-muscular cells may occur: it is possible that the neurites may touch very many epithelio-muscular connexions during the intracellular space, besides making contact with the muscle-sheet at the end plate.

The sensory system

The plan of the nerve-net has been discussed by one of us on an earlier occasion (Pantin, 1952). Less has been said about the sensory system. Actinian sensory cells from a variety of species were first described by the Hertwigs (1879), particularly from cells isolated by maceration. Our results confirm the general form of the cells which they describe.

In *Metridium*, the silver technique we employed, while it shows the nerve-cells well, never stains the sense-cells. Reduced methylene blue, however, shows the sensory cells in pieces of anaesthetized tissue prepared in the manner described. Better still, reduced dye injected through the body-wall of a partially anaesthetized and expanded animal gives successful results.

As the Hertwigs showed, the sense-cells are 'primary sense cells', like the olfactory cells of vertebrate histology. Such cells can be seen scattered over the surface of the mesentery, sparsely as in fig. 2 or numerously as in fig. 9, A. Fig. 9, B shows such a primary sense-cell fixed by the Susa method after vital staining with methylene blue. It is of interest that the entire cell stains in this way, including the flagellum. The cell-body is often surmounted by a flagellum which may reach 25 μ. Commonly, however, it appears short as in fig. 9, B and is sometimes absent. Though that may be due to breakage, McConnell (1932) records similar short flagella in the sensory cells of *Hydra*.

The cell-body is 10 to 24 μ long and stands in the epithelium. It varies considerably in extent and in shape in accordance with the state of extension of the epithelium. Often the upper part of the cell-body is curved over in characteristic 'umbrella-handle' as in fig. 9, B. This may be due to the top part of the cell being pulled by the shifting epithelial surface.

From the base of the cell-body arise one, two, or rarely three fine tapering processes (figs. 2, 9, 10) which may run at least several hundred μ. Provisionally these processes will be termed sensory 'neurites'. It has not been possible to follow them to their termination, and though they lie near the muscle-sheet it is not known whether they make contact with the muscle-fibres. The neurites, however, certainly make contact with each other and with the neurites of the main bipolar nerve-net (figs. 10, B, C). Havet (1901),

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FIG. 8 (plate). A variety of neurite terminations over the retractor muscle-field, taken from silver-stained whole mesenteries. Muscle-fibres and epithelial nuclei are also seen.

using the Golgi method and also methylene blue on small *Metridium*, showed similar sensory cells and traced their connexions with the nerve-net. Groselj (1909) showed the abundance of sensory cells and of other connexions without anastomosis, particularly in *Bunodes* and *Cerianthus*, by vital staining with methylene blue. Wolff (1903) also found contact between sensory cells and the nerve-net in *Heliactis* (= *Cereus*), though unlike Groselj and Havet and ourselves he believed that there was protoplasmic continuity existing throughout.

Where they are numerous, the fine neurites of the sense-cells may form an extensive net above the muscle-sheet (figs. 9, C; 10, A). This net has never been seen to penetrate below into the mesogloea.

The numbers of the sensory cells vary considerably in different regions of actinians, as Groselj (1909) showed. In *M. senile* we found few over much of the surface of the mesentery (see fig. 2), but great numbers are collected where the mesenteries are attached to the body-wall (figs. 9, C; 11) or to the pedal disk. In these regions the fine network of neurites is particularly well seen. Fig. 9, C shows a mesentery turned on its side against the body-wall of *Metridium*. The abundant sensory cells and the network formed by their neurites can be seen. The distal part of the mesentery has been cut away.

Organization of the nerve-net and sensory system

In this paper we are particularly concerned with the nerve-net of the mesenteries and with indications of its connexions. The examination of the oral disk, the pharynx, and the net in the marginal sphincter will be reserved for later work. On many essential points our work confirms and extends that of the Hertwigs (1879). The chief points of difference in our account from that of some previous ones concern (1) the non-syncytial synaptic character of the junctions between the neurites both of the nerve-cells and of the sensory cells (this has already been discussed (Pantin, 1952)); (2) the wholly intra-epithelial character of this system, at least in the mesenteries and the column. We have found no trace of nerve-cells or neurites in the mesogloea of this region, and we shall show in a later paper that there is reason to attribute many records of mesogloeal nerve-fibres in actinians to one or other of a variety of artifacts, as was indicated in an earlier paper by one of us (Pantin, 1952).

Fig. 12 summarizes our conclusions. As the Hertwigs showed, the network of the neurites of both sensory cells and nerve-cells runs between the epithelial part of the cells and the underlying muscular sheet. The system differs from that of triploblastic animals (*a*) in the purely intra-epithelial character

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FIG. 9 (plate). Sense-cells from methylene-blue preparations.

A, numerous sense-cells in a mesentery near its junction with the body-wall. Fixed with ammonium molybdate.

B, sense-cell from a mesentery (note that the flagellum has stained). Fixed with Susa / phosphomolybdic acid.

C, living methylene-blue preparation showing aggregation of sense-cells on both sides of a mesentery at its junction with the body-wall. The dark line is the cut edge of the mesentery and the pale strip its line of attachment. Note sensory axons (between arrows).

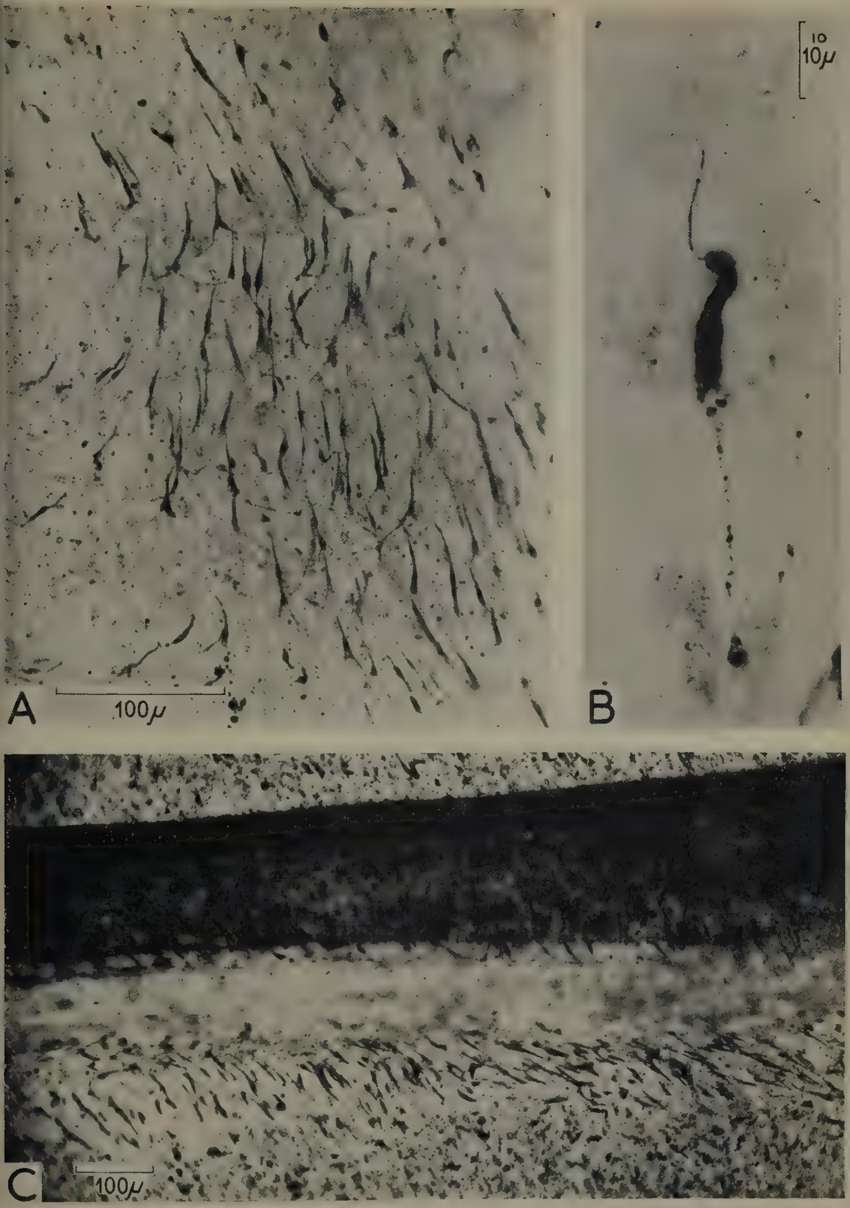

FIG. 9

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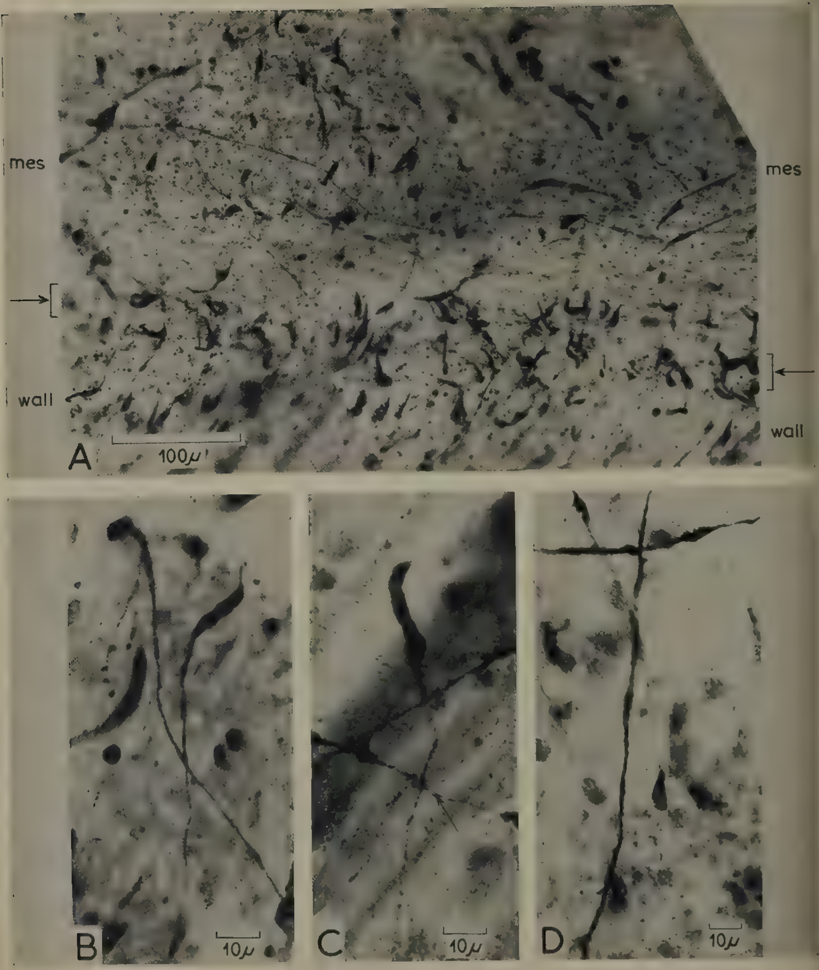

FIG. 10

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mentioned above, and (*b*) in that like the muscle-sheet it is essentially two-dimensional (Pantin, 1960).

Another feature of the nerve-net neurites is the absence of sheath-cells such as are found round axons and nerve-bundles in the non-medullated nerves, for example, of insects and vertebrates (Edwards and others, 1958). On the other hand, their appearance recalls that of the growing neurites in cultures of embryonic chick nervous tissue (Harrison, 1910; Hughes, 1953). These neurites may grow out for long distances in a linear manner. Where they cross they make contact but do not fuse, as is clearly indicated in time-lapse films by the visible movement of material along each separate axon. On fixation, chick neurites in old cultures often appear beaded in the same fashion as in the actinian nerve-net neurites. A time-lapse film taken by Dr. A. Hughes, which he kindly lent us together with one taken by K. Hayden and C. M. Pomerat ('The behaviour of embryonic chick spinal cord in tissue culture'), shows the amoeboid advancing ends of growing neurites to have terminal expansions reminiscent of the endings of the neurites of the actinian nerve-net. The time-lapse films of advancing chick neurites also show that the amoeboid processes of the advancing tip from time to time make temporary contacts with adjacent neurites which may remain as twig-like branches. These recall the short twig-like branches seen on actinian neurites (fig. 7, c).

The actinian nerve-net may thus be likened to a neurite tissue culture in which the axons are developed in a plane between epithelium and muscle-layer. Each neurite repeatedly crosses others in its path and adheres without syncytial fusion to those with which it comes in contact.

The sensory component of the nerve-net consists of simple sense-cells. As the Hertwigs showed, each consists of a cell-body with a distal flagellum, and one, two, or three fine basal neurites. Their function is known only by inference. Apart from their connexion with the nervous system, they closely resemble the elementary sensory cells of higher animals. They also resemble those of Medusae; and in these animals the local aggregation to form sense-organs leaves no doubt of their function. Despite specialized responses, particularly by the oral disk, to different classes of sensory stimuli such as mechanical, chemical, and light (Pantin and Pantin, 1943; North and Pantin, 1958), the sense-cells of *Metridium* all seem essentially similar and show no evident structural differentiation into classes, except possibly in the length of the flagellum.

FIG. 10 (plate). A, living preparation of body-wall, stained with methylene blue. The arrows indicate a row of sense-cells where a mesentery joins the body-wall. Above this is the mesentery (*mes*), in which further sense-cells and nerve-cells are seen. The area below the arrows is the body-wall (*wall*).

B, junction between the neurites of two sense-cells (near the base of a mesentery). Methylene blue, fixed with ammonium molybdate.

C, junction between the neurite of a sense-cell and that of a nerve-cell (indicated by an arrow), from a mesentery stained with methylene blue. Fixed with Susa / phosphomolybdic acid.

D, termination of a neurite stained with methylene blue, showing expanded ending over the muscle-field. From a whole mesentery (fixed with Susa / phosphomolybdic acid).

There are no complex sense-organs in sea-anemones. Hollard (1851) had made a suggestion that the acrorhagi of *Actinia equina* might be sensitive to light. Schneider and Rötteken (1871) and Duncan (1874) interpreted their retractile aggregates of nematocysts as lenses of a sort of compound eye. Korotneff (1876), Jourdan (1880), and Béranek (1888) repeated this. But more recently Abel (1954) has shown that the acrorhagi are weapons of defence; they can be inflated and extended against neighbouring animals.

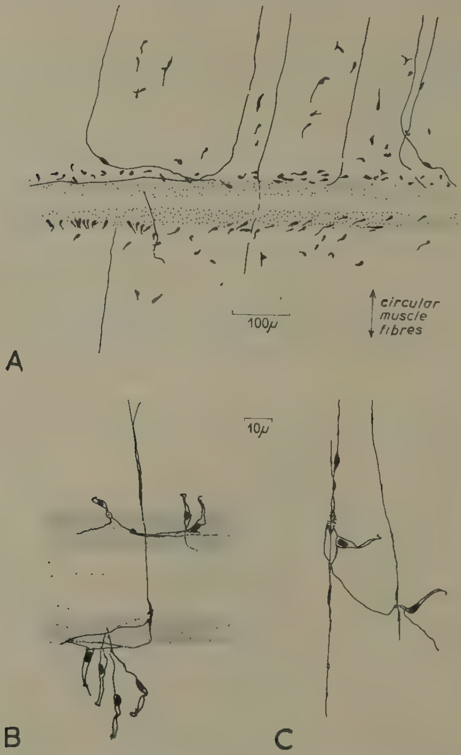

FIG. 11. Camera lucida drawings of a body-wall preparation stained with methylene blue. The circular muscle-field runs north–south, as shown by the arrow. A, neurites follow the direction of circular muscle-fibres in the body-wall, and on reaching a mesentery, either follow the circular fibres beneath it or turn through 90°. The insertion of the mesentery is shown by stippling. B, detail from an area adjacent to A. Two neurites follow the circular muscle-fibres beneath the insertion of the mesentery. A few of the numerous sense-cells are shown. C, detail from the body-wall, showing connexions of two sense-cells with neurites. Some beading has occurred.

The numbers of the sense-cells vary significantly in different parts of the body. As might be expected, they are very numerous in the tentacles and oral disk and particularly so in the oesophagus and the mesenterial filaments (Hertwig and Hertwig, 1879; Groselj, 1909). They are fairly numerous in the pedal disk and sparse in the ectoderm of the column. As Passano and Pantin (1955) showed, this ectoderm may be removed without reducing at all the animal's sensitivity to mechanical stimulation. A few sense-cells are scattered over the general surface of the mesenteries and the general endoderm of the column, but they are very numerous in the parietal region, where the mesenteries join the body-wall. Movements of the tissue inevitably cause mechanical deformation of the sensory cells, and the aggregation of the latter in the parietal region is peculiarly suitable for sensitivity to mechanical stimulation of the body-wall. Passano and Pantin (1955) pointed out that mechanically the whole column system resembles a gigantic Pacinian corpuscle. Possibly also the sense-cells serve for some kind of proprioception during the

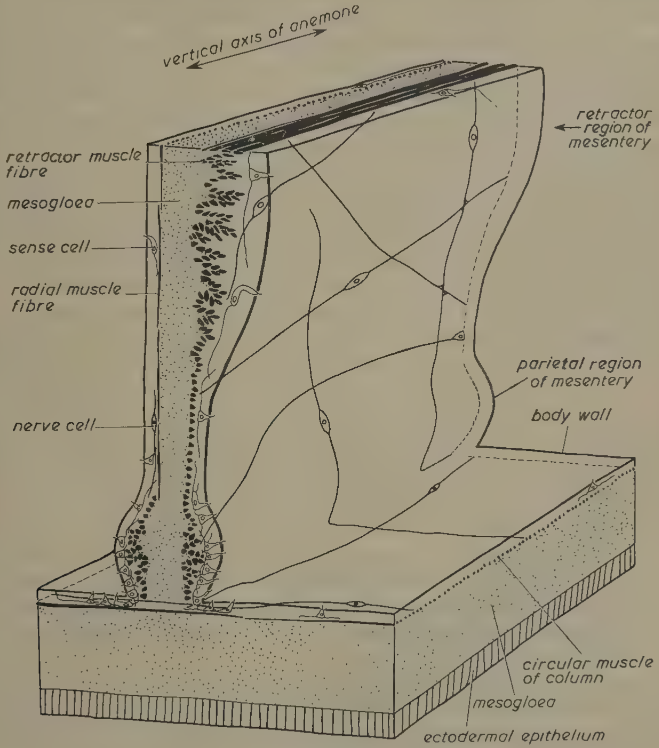

FIG. 12. Diagrammatic summary of the histological elements of the mesentery and body-wall which have been discussed.

movements of the animal. These movements are co-ordinated to give the animal a variety of functional shapes (Batham and Pantin, 1950). The ease with which mechanical excitation produces the retraction reflex even in parts of the column isolated by cuts shows that the sensory elements are widely distributed. The general distribution of sensory cells and their contacts with the nerve-net are in accordance with these properties. The considerable aggregation of sense-cells at the base of the mesentery is of interest because it is here that the antagonistic muscle-systems of the parietal and circular muscle-fields are in apposition.

Passano and Pantin (1955) brought evidence to show that during mechanical stimulation graded excitation was set up in the sensory system which generated impulses in the nerve-net. They pointed out the difficulty of supposing that these arise in the bodies of the sense-cells and that experiment suggested that the responding element was a system tangential to the body-wall. In this situation, the fine network of sensory neurites would supply just such a system when distorted by movements of the tissues (see fig. 9).

The sense-cell bodies with their flagella may well correspond to the chemo-sensitive elements which undoubtedly exist. Indeed, the same sense-cell may perhaps respond to different kinds of stimuli. Pantin (1935 *a*) and Pantin and Pantin (1943) pointed out that continual electrical excitation of the nerve-net of the oral disk could produce a rejection reaction if not prolonged, or a feeding response if a long succession of shocks was given. Mechanical stimulation shows rapid adaptation whilst that to chemical stimulation is slow. Mechanical excitation via the neurites of the sense-cells and chemical via the flagellate cell-body might account for such a difference.

The sensory cells and their axons make synaptic contact with the general nerve-net. It has been pointed out that this is intra-epithelial and does not traverse the mesogloea. These facts are in full agreement with the physiological evidence. Thus, the mesenteries are functionally quite independent effector systems (Batham and Pantin, 1950). The separation of the nerve-nets on the two surfaces agrees with this.

The mesenteric nerve-net, particularly the well-developed net on the retractor surface, makes contact with the net of the general body-wall endoderm. This sparser net with the tendency of its neurites to run parallel with the circular muscle-sheet (fig. 11) agrees with the limited vertical conduction in the body-wall.

Through-conduction and the nerve-net

Let us first consider the physiological evidence to which this histological picture relates. The early experiments of Parker (1919) on *Metridium* showed that whereas in certain regions, such as the disk and the tentacles, the response to excitation was localized and decreased rapidly with the distance from the stimulus, stimulation at any position on the column resulted in symmetrical excitation of all the responding muscles in the retraction reflex. His experiments show the existence of a through-conduction system in this region serving the muscles. Pantin (1935 *a*) showed that a similar system obtained in *Calliactis parasitica*, and that there was here a well-defined threshold followed by a refractory period; and that all the evidence is consistent with a brief electric shock initiating an all-or-nothing nervous impulse which is conveyed to the responding muscles. There is evidence that even a single impulse traverses the through-conduction system, though it does not excite contraction. Experiments with pairs of stimuli show that for a single successful stimulus of brief duration there is one, and only one, block in the pathway between the point of stimulation and the responding muscle. Pantin (1935 *a*, *d*) noted the evidence that this block occurred between the through-conduction system and the contractile system itself. A single stimulus, though apparently unsuccessful, nevertheless facilitates the transmission of a second from the through-conduction system to the contractile system. Hall and Pantin (1937) showed that many of the characteristics of excitation of *Calliactis* were exhibited in *M. senile*, the same genus with which Parker worked. Similar through-conduction and facilitation phenomena were found in a tropical species of *Bunodactis* (Pantin and Vianna Dias, 1952 *a*).

In all these species, excitation at any point of the column may call the retractors, the marginal sphincter, or the parietals into action according to the number and frequency of the stimuli. The threshold for all these responses is identical. Subthreshold stimulation is without effect.

The system of through-conduction, with facilitation between this and the contractile system, has a close parallel in Scyphomedusae (Bullock, 1943; Pantin and Vianna Dias, 1952 *b*).

By drastic cutting of the body-wall of *Metridium*, Parker showed that excitation leading to retraction was conducted in every direction through the column. It was not confined to specific tracts, and there was no evidence of polarized direction of conduction. He did, however, show that conduction took place more readily up the mesenteries than horizontally round the body-wall. Pantin (1935 *b*) did comparable experiments on *Calliactis* using electrical stimulation and measured the conduction velocity in different parts of the system. In conformity with Parker's qualitative experiments, he found ready conduction up the mesenteries; their speed of conduction is much higher than round the body-wall, except in the neighbourhood of the marginal sphincter.

The physiological evidence would thus lead us to expect the existence of the nerve-cells running up the mesenteries and making contact with the retractor muscles, and to expect that any such system of nerve-cells would be unlikely to show anatomical polarization in its organization or in the structure of its synaptic functions. We should, however, seek the anatomical site of neuromuscular facilitation between the nerve-cells and the muscle-fibres (Pantin, 1952).

The histological evidence presented in this paper agrees well with these requirements. As we have seen, running up the retractor surface of the mesenteries is a well-developed network of bipolar nerve-cells. The network shows no histological evidence of junctional polarization. The system closely resembles that of the through-conduction system of the scyphozoan umbrella. In that, Horridge (1954) has demonstrated very beautifully that a single large bipolar cell of the sub-umbrella net is necessary and sufficient to conduct excitation and that this is accompanied by an electrical impulse of the usual kind.

The retractor nerve-tract runs on the retractor face of the mesenteries and extends over the parietal muscles. Its relation to the sphincter will be discussed in a later paper. We have seen that it is in continuation with a much sparser system of neurites, with fewer contacts per unit area, over the circular muscle-sheet of the general body-wall. These anatomical features are consistent with the ability of the through-conduction system to activate the parietals, and to conduct more slowly and with more probability of failure in the column wall as opposed to the mesenteries (Parker, 1919; Pantin, 1935 *b*).

We have already noted that the separation of the well-developed nerve-tract of the retractor surface from the weakly developed one of the radial surface of the mesentery is in keeping with the physiological independence of the muscle-systems of the two faces (Batham and Pantin, 1950). The radial musculature is not activated by the excitation of the through-conduction system.

Pantin and Vianna Dias (1952 *a*) showed that in some anemones there is evidently complication of the parietal system. In *Bunodactis* excitation of the through-conduction system causes typical slow symmetrical parietal contraction. But mechanical stimulation causes rapid local contraction on the side touched. There appears to be more than one kind of excitation system in the parietals of these animals, or else the parietal muscle-sheet itself may be differentiated into two parts only one of which is connected to the through-conduction system.

In considering the organization of the nerve-net on the retractor face of the mesentery, it should be borne in mind that in this paper we are solely concerned with the nerve-net in the region which, physiologically, is regarded as a through-conducting system. In the disk and elsewhere the physiological properties are different and the histology of the nerve-net may be different.

Muscular excitation

The retractor muscle is covered with the network of neurites. Over most of their course these run in the intercellular space above the muscle-fibres. The expanded, often plate-like endings are commonly at a lower level, and seem to be actually in contact with the muscle-sheet (fig. 8). The precise nature and extent of this contact await electron-microscopical study. Although there is as yet no direct evidence as to their function it would seem natural to suggest that these endings may be the site of excitation of the muscle-sheet by the through-conduction nerve-network. They are suitably placed anatomically, and there are no alternative structures which could obviously fulfil that function.

It should be remembered, however, that throughout their course in the intercellular space the neurites are passing through a forest of strands connecting the muscle-fibres to the epithelial parts of the cells above. Excitation through these connecting strands cannot be ruled out.

The hypothesis that excitation in the histologically visible nerve-net causes facilitated contraction in the underlying muscle-sheet is adequate for simple responses in muscles such as the marginal sphincter of *Calliactis* (Pantin, 1935 *a*, *d*) and the retractor of *Metridium* (Hall and Pantin, 1937). In these muscles each facilitated contraction follows the stimulus in about 50 to 80 msec, most of which can be shown to be due to conduction time in the through-conduction system. Each single contraction step reaches its maximum tension in a few tenths of a second. It is for these contractions that inferences can certainly be drawn about the nature and site of the facilitation. But there are two features of this simple interpretation which need to be examined. Some actinian muscles, notably the retractors of the mesenteries and sphincter, can give two distinct kinds of contraction, the quick contractions we have been discussing and also a much slower one. Observation of isolated mesenteries under the microscope indicates that the whole retractor field is capable of both kinds of contraction. Both are initiated via the through-conduction system; but the fast one requires relatively higher frequencies. The second feature that ultimately needs explanation is that, though connected with the through-conduction system, the parietal muscles sometimes show reciprocal inhibition in relation to the circular muscle of the column (Batham and Pantin, 1954).

Let us consider first the nature of the slow contraction. These authors found this in a variety of actinian muscles. It is a very slow smooth sigmoid rise in tension, preceded by a variable latent period about 1,000 times as long as that for typically facilitated responses. It can be produced in a variety of the muscles such as the parietals of the body-wall, by excitation of the through-conduction system at very low frequencies such as 1 shock per 5 sec, which is below the range characteristic for clearly facilitated contractions (generally less than 1 in 2 sec). It was first supposed that these slow contractions were comparable to the quick facilitated ones in which, however, the step-like increments of the contraction were smoothed out in a very 'viscous', slowly acting muscle (Pantin, 1935 *a*). Batham and Pantin (1954) showed that this explanation would not do and that the slow contractions differed in certain important features from the simple facilitated responses.

All actinian muscles so far investigated can give the slow kind of contraction in which characteristically the rise in tension may take a minute or more. Only some of the muscles, including the retractor, sphincter, and disk radials of *Metridium* and the sphincter of *Calliactis*, can also give a much more rapid contraction in which the rise in tension is accomplished in a few tenths of a second. Quick and slow contractions are common in invertebrate muscles. But they have so far always been correlated with the existence of two sorts of muscle-fibre or of two kinds of motor innervation.

In the actinians there is as yet no histological evidence from any source of multiple innervation of a given muscle. Nor is there any histological differentiation of the muscle-fibres into two distinct classes (as Batham and Pantin (1954) pointed out). Indeed, electron micrographs (Grimstone and others, 1958) confirm that there is no difference between the ultimate fibrillar structure of the muscle-fibres which can only contract slowly and those which contract swiftly as well. But those muscle-fibres which can contract rapidly as well as slowly are decidedly larger in both length and breadth than those which can only contract slowly. This can be seen in the length of the retractor fibres as against that of the slow radial fibres in fig. 5.

The fibres of muscles which can contract both quickly and slowly are as much as 10 times the linear dimensions of those that only act slowly. In the mesenteries, the number of epithelial cell-bodies per unit area is, nevertheless, much the same over both kinds of muscle. Since each cell is connected to one muscle-fibre, the numbers of muscle-fibres on each surface must also be similar. The hypertrophied muscle-sheet in the retractors is thus made up of muscle-fibres which, although longer and broader than, for example, those of the radial muscle of the mesentery, are not more numerous. This increased length of the enlarged muscle-fibres gives rise to a higher density of fibres per unit area. Muscular hypertrophy is accompanied by increase in numbers and diameter of nerve-cells over the retractor: the probability of innervation of the muscle-fibres is increased.

On the other hand, we have seen that the muscle-sheets which only act slowly, like the circular muscle of the body-wall and the radial muscle of the mesenteries, have very small muscle-fibres and very sparse innervation. Inspection of figs. 5, A and 6, A shows that very large numbers of the radial muscle-fibres receive no evident innervation at all. They could only execute a contraction initiated from the neurites if contraction is recruited by spreading through the muscle-sheet.

In contrast, consideration of the richness of the nerve-net on the retractor face and the greater length of the muscle-fibres in fig. 5, B shows that most if not all muscle-fibres will be traversed by a neurite, whatever may be their supposed site of physiological contact. Consequently, in this case excitation may be able simultaneously to activate many points over the whole retractor muscle. Slow contraction in this same muscle might be produced by excitation at a few sites only in the net. Until this hypothesis is tested the presence of two kinds of contraction in the same muscle does not enforce a decision that there must be two histologically separate systems of muscular innervation.

The apparent occurrence of reciprocal inhibition between the parietal and circular muscle-fields (Batham and Pantin, 1954) cannot at present be explained in terms of simple neuro-muscular organization exhibited histologically. It will be recalled, however, that Horridge (1955) has clearly demonstrated inhibition of nervous activity in several Hydromedusae.

Apart from excitation by way of the nerve-net, the muscular system of actinians may be accessible to direct stimulation (Pantin, 1956; North and Pantin, 1958). Coelenterate muscle-cells might also be accessible to direct excitation from the sense-cells. Parker (1919) suggested the existence of direct connexion between the sensory cells with their processes and the muscle-fibres, but no direct evidence of this has yet been produced. The long fine neurites running through the intercellular space from the sense-cells, like those of the nerve-cells, presumably must touch some of the cytoplasmic strands connecting the upper and lower components of the epithelio-muscular cells. But the nature of these contacts is unknown. Hadži (1909) found in teased preparations from *Hydra* occasional sensory cells adjoining endodermal epithelio-muscular cells, but considered that this might be due to casual adhesion. The difficulty with all such coelenterate sensory cells is that their dissemination amongst other cells and the fineness of their neurites make the ultimate terminations exceedingly difficult to determine.

The sensory and nervous systems of the mesenteries and column and their anatomical relation to the responding muscles agree well with the physiological properties of the retraction reflex. It is when we bear in mind that this is but one specialized member of the set of complex activities of which actinian behaviour is composed that one is surprised at the apparent lack of any histological evidence of further complication of the nervous and muscular system.

Much of this work was done at the Marine Biological Laboratory, Plymouth, to the Director and staff of which we are most grateful for the many facilities given us. We wish to thank the Department of Scientific and Industrial Research for grants for the development of a special research which enabled this work to be carried out, and the Council of Trinity College, Cambridge, for a contribution to the cost of the plates.

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